BIOELASTICS RESEARCH, LTD

DEVELOPMENT OF BIOELASTIC MATERIALS FOR THE PREVENTION OF ADHESIONS: POLYPENTA- AND POLYTETRAPEPTIDES

UNITED STATES NAVY
CONTRACT NUMBER N00014-89-C-0282

FINAL REPORT

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for the Prevention of Adhesions: Polypenta- and Polytetrapeptides

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A. D. W. Urry, "Preprogrammed Drug Delivery Systems Using Chemical Triggers for Drug Release by Mechanochemical Coupling," Am. Chem. Soc., Div. Polym. Mater.: Sci. and Eng. **63**, 329-336, 1990.

B. D. W. Urry, "Bioelastic Materials as Chemomechanically Transducing ("Smart") Matrices for Drug Delivery," In Cosmetic and Pharmaceutical Applications of Polym. (C. G. Gebelein, T. C. Cheng, and V. C. Yang, Eds.), Plenum Press, New York, 181-192, 1991.

C. Dan W. Urry, Timothy M. Parker, Michael C. Reid, and D. Channe Gowda, "Biocompatibility of the Bioelastic Materials, Poly(GVGVP) and Its γ–irradiation Cross-linked Matrix: Summary of Generic Biological Test Results," J. Bioactive Compatible Polym. **6**, 263-282, 1991.

D. Dan W. Urry, C-H Luan, T. M. Parker, D. C. Gowda, K. U. Prasad, M. C. Reid, and A. Safavy, "Temperature of Polypeptide Inverse Temperature Transition Depends on Mean Residue Hydrophobicity," J. Am. Chem. Soc. **113**, 4346-4348, 1991.

E. Frederick J. Elsas, D. Channe Gowda and Dan W. Urry, "Synthetic Polypeptide Sleeve for Strabismus Surgery," J. Pediatr. Ophthalmol. Strabismus **29**, 284-286, 1992.

F. D. W. Urry, A. Nicol, D. C. Gowda, L. D. Hoban, A. McKee, T. Williams, D. B. Olsen and B. A. Cox , "Medical Applications of Bioelastic Materials," In Biotechnological Polymers: Medical, Pharmaceutical and Industrial Applications (Charles G. Gebelein, Ed.), Technomic Publishing Co., Inc., Atlanta, Georgia, 82-103, 1993.

G. Dan W. Urry, D. Channe Gowda, Betty A. Cox, Lynne D. Hoban, Adam McKee and Taffy Williams "Properties and Prevention of Adhesions Applications of Bioelastic Materials," Mat. Res. Soc. Symp. Proc. **292**, 1993 (in press).

H. Lynne D. Hoban, Marissa Pierce, Jerry Quance, Isaac Hayward, Adam McKee, D. Channe Gowda, Dan W. Urry and Taffy Williams, "Use of Polypentapeptides of Elastin to Prevent Postoperative Adhesions: Efficacy in a Contaminated Peritoneal Model," J. Surgical Res. 1993 (in press).

I. Alastair Nicol, D. Channe Gowda, Timothy M. Parker and Dan W. Urry, "Elastomeric Polytetrapeptide Matrices: Hydrophobicity Dependence of Cell Attachment from Adhesive, $(GGIP)_n$, to Non-adhesive, $(GGAP)_n$, Even in Serum", J. Biomed. Mtrls Res., 1993 (in press).

Development of Bioelastic Materials
for the Prevention of Adhesions: Polypenta- and Polytetrapeptides
Contract No. N00014-89-C-0282

FINAL REPORT
(September 15, 1989 - March 14, 1993)

I. PURPOSE OF THE CONTRACT

The general purpose of the contract was the development of bioelastic materials for the application of preventing post-surgical and post-trauma adhesions by preparing compliant sheets which can be placed between repair sites and which act as barriers to the formation of adhesions between the repairing sites. Specifically to be prepared were quantities of a series of polypentapeptides and polytetrapeptides which were γ–irradiation cross-linked to form elastomeric sheets and which were supplied to the Naval Medical Research and Development Command, Combat Casualty Care Unit for the animal model efficacy studies carried out at the Naval Medical Research Institute. In addition, in the process of carrying out the specific objectives, consideration of further applications was stimulated and these stimuli, coming from interactions with Navy personnel and others, have resulted in the development of intellectual property with the support of this contract.

II. SUMMARY OF MATERIALS PREPARED AND SHIPPED

The contract called for the production of 5-gram quantities of each of six preparations. A seventh preparation involving 2 grams was added later. These preparations are as follows:

Preparations 1 & 2: In excess of 10 grams of poly(VPGVG) were prepared and cross-linked to form fifteen 3" x 3" sheets. Approximately one-half were used directly and the others were heat-treated to form State III before using in the animal models.

Preparation 3: In excess of 5 grams of poly(IPGVG) were synthesized to produce eight cross-linked sheets.

Preparation 4: Over 5 grams of poly[3(VPGVG),(VPGFG)] were synthesized and cross-linked to form eight sheets.

Preparation 5: Over 5 grams of poly[9(VPGVG),11(VPGAG)] were synthesized and cross-linked to form eight sheets.

Preparation 6: Quantities of (VPGVG) and VPGEG) were synthesized such that over five grams of poly[4(VPGVG,(VPGEG)] were produced.

Preparation 7: Approximately 2 grams of poly(VPAVG) were synthesized and cross-linked to form three sheets.

Additionally, 800 mg of poly(VPGVG) and 800 mg of poly(IPGVG) were provided to Dr. Hoban in sterilized syringes for use in animal model studies.

The contract also called for the production of 4 gram quantities of each of 5 polytetrapeptide preparations. These are listed as polymers IX, X, XI, XII and XIII of Table I and shipment dates are indicated therein.

The above represent the major shipments on this contract. Additional materials were supplied thereafter, and are included in Table I (pages 8 & 9), which summarizes all the materials which were prepared and shipped to the Naval Medical Research and Development Command, Combat Casualty Care Unit.

Upon determination that the Poly(GGAP), even in the presence of serum, exhibits no cell adhesion properties, as shown in Figures 1, 2 and 3 below, the decision was made to provide this material in syringes to NAmSA® for biocompatibility testing. In Figures 1, 2 and 3, LNF stands for ligamentum nuchae fibroblasts, HUVEC for human umbilical vein endothelial cells, FBS for fetal bovine serum, BSA for bovine serum albumin, and TC for tissue culture plastic (–0 without coating and –hFN coated with human fibronectin).

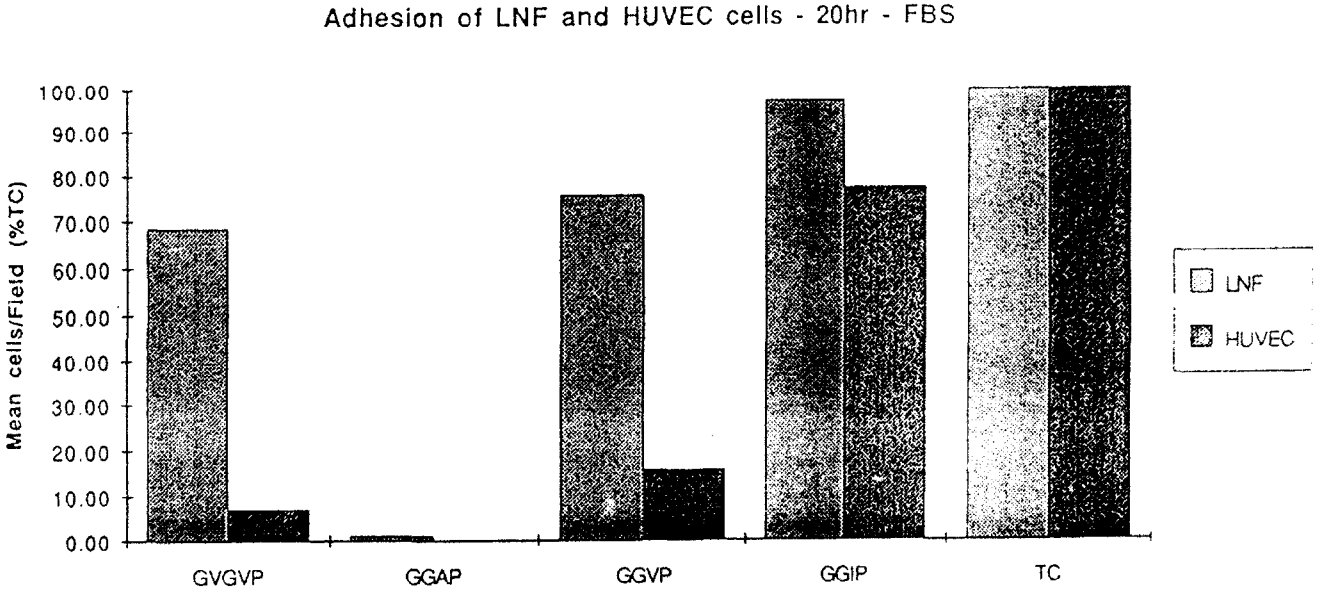


FIGURE 1

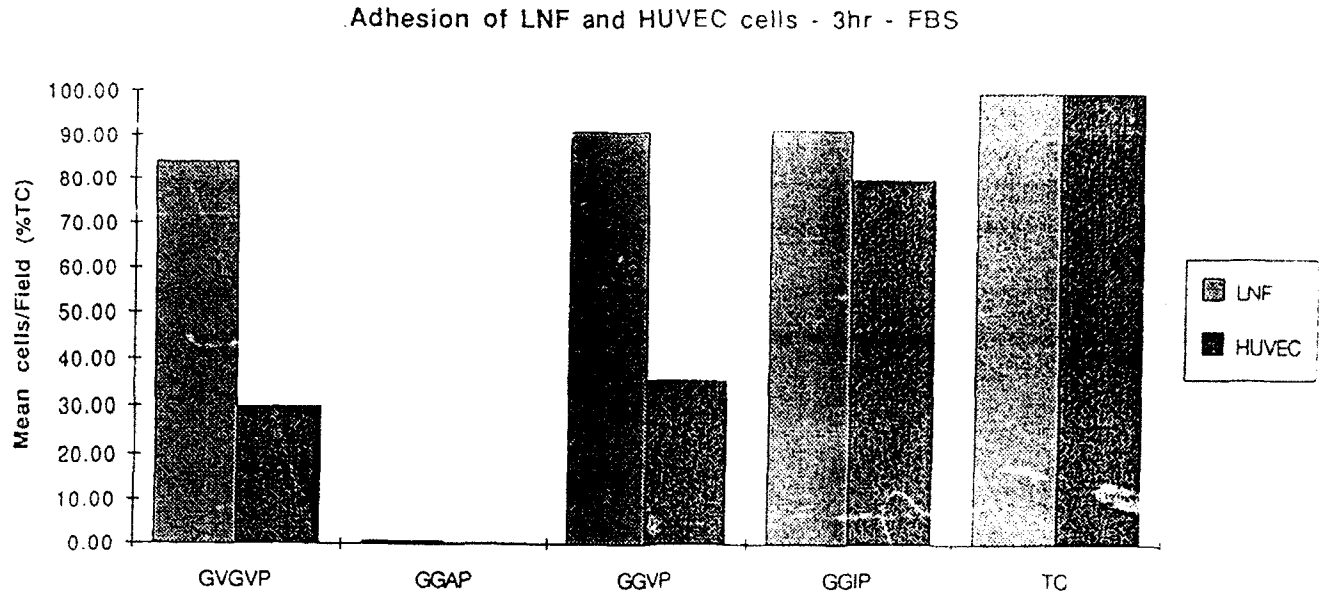


FIGURE 2

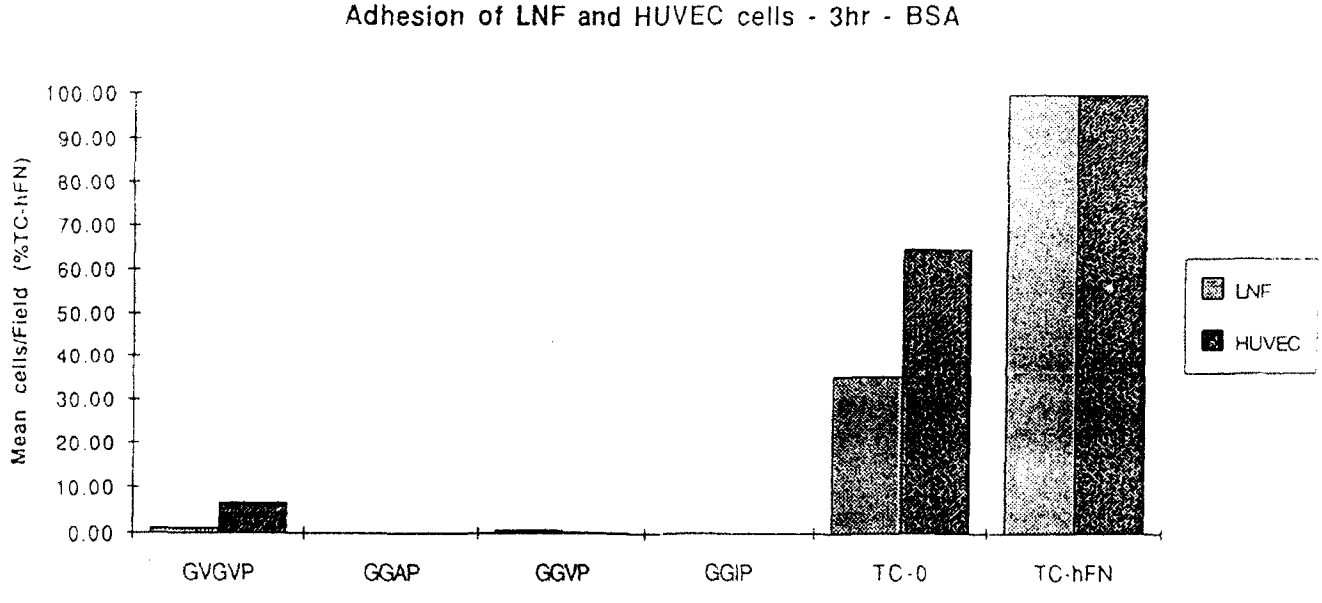


FIGURE 3

III. PHYSICAL CHARACTERIZATIONS OF MATERIALS

One of the physical characterizations obtained for the polymers is the determination of T_t. Below a certain temperature, each of the polymers is soluble in water. Upon raising the temperature, there occurs an onset of aggregation resulting in a phase transition to form a more-dense, polymer-rich, viscoelastic state called a coacervate. The onset of the transition can be followed spectrophotometrically by the temperature profile of turbidity formation where the temperature for half-maximal turbidity is designated as T_t. These T_t values have been included In Table I (pages 8 & 9).

Additionally, if the polymer contains a functional side chain such as Glu(E), the T_t is dependent upon the pH value of the polymer. In Figure 4 an acid-base titration of Poly[9(GVGVP),1(GEGVP)] is shown with a resulting pK_a value of 4.4. In Figure 5 an acid-base titration of Poly[9(GGIP),1(GEIP)] shows a pK_a value of 4.5.

T_t for Poly[9(GVGVP),1(GEGVP)]

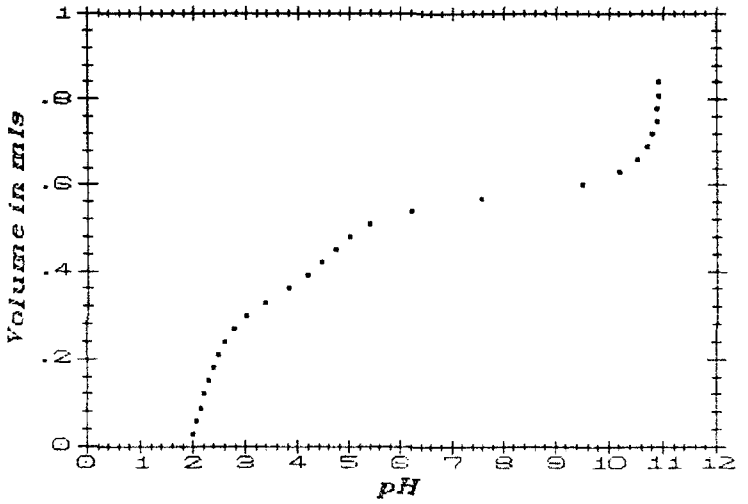


FIGURE 4

T_t for Poly[9(GGIP),1(GEIP)]

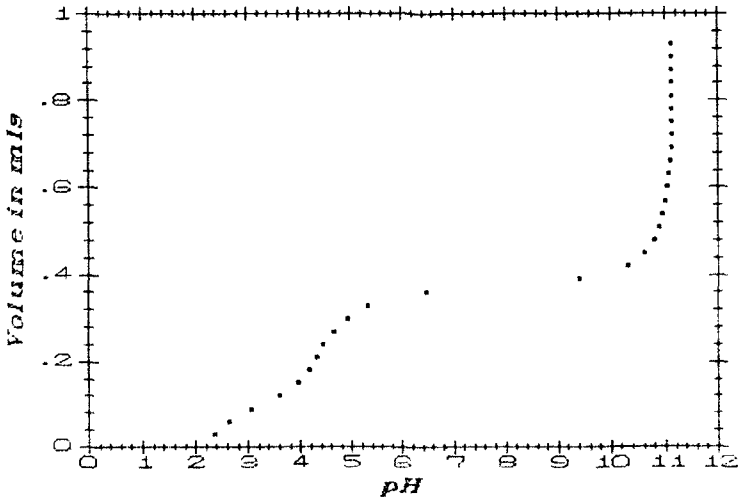


FIGURE 5

Figures 6 and 7 demonstrate the effects of pH on the T_t value in phosphate buffered saline (0.15 N NaCl, 0.01 M phosphate). Figures 4 through 7 were included as representative physical characterizations necessary for the proper design and development of these materials for the prevention of adhesions application.

Tt Dependence on pH
for Poly[9(GGIP),1(GEIP)]

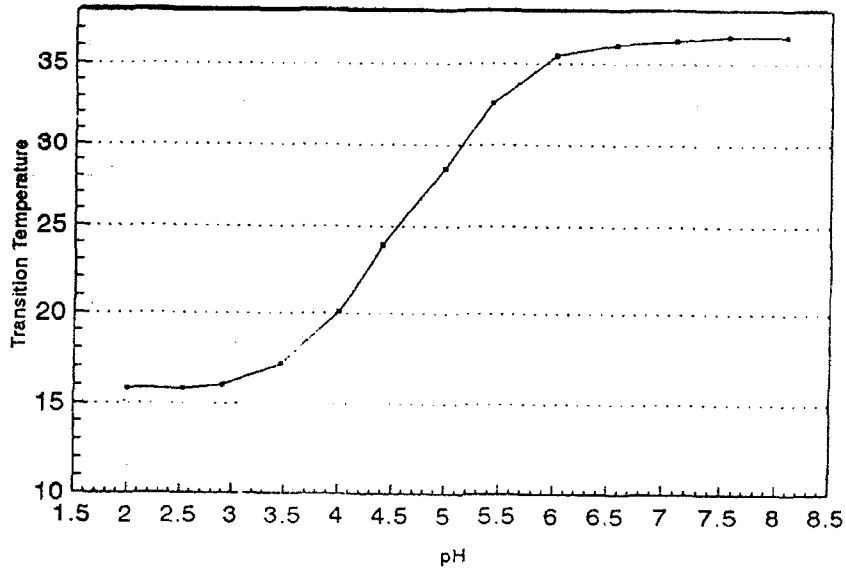


FIGURE 6

Tt Dependence on pH
for Poly[9(GVGVP),1(GEGVP)]

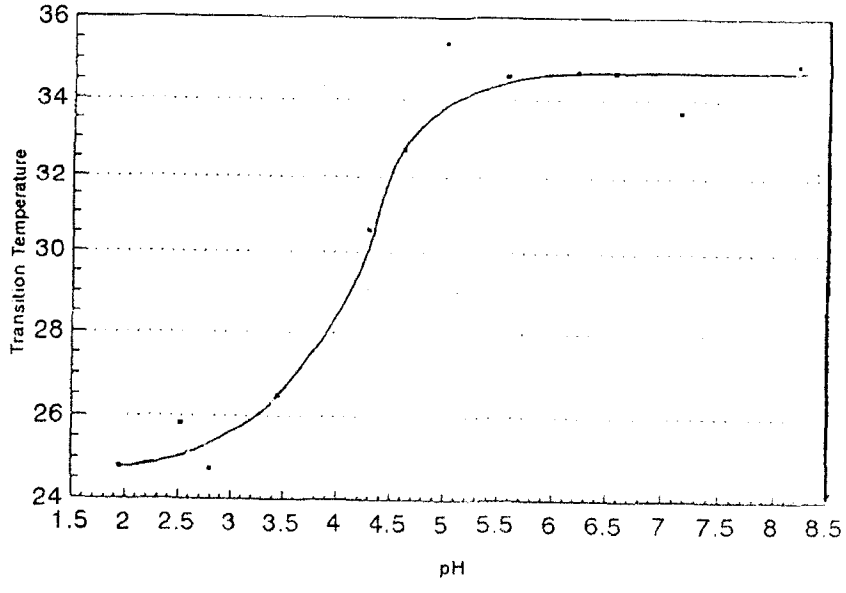


FIGURE 7

TABLE I

"Development of Bioelastic Materials for the Prevention of Adhesions; Polypenta– and Polytetrapeptides"
N00014-89-C-0282
DATES: 9/15/89 - 3/14/93

Summary of Materials Prepared and Shipped

POLYMER	QUANTITY PREPARED	T_t	DATE SHIPPED	CONFIGURATION & PACKAGING
I: Poly(VPGVG)	10 gms	24.8°C	5/14/90, 6/6/90	15 Patches in Teflon Sheets
II: Poly(IPGVG)	5 gms	12.8°C	08/13/90 08/29/90	6 in Teflon Sheets 2 in Petri Dishes
III: Poly[3(VPGVG),(VPGFG)]	5 gms	12.2°C	06/25/90 08/13/90	7 in Teflon Sheets 1 in Teflon Sheet
IV: Poly[9(VPGVG),11(VPGAG)]	5 gms	43°C	08/29/90 08/29/90	2 in Petri Dishes 4 in Sterile Tubes
V: Poly[9(VPGVG),1(VPGEG)]	5 gms	Fig. 4	07/09/91	8 in Sterile Tubes
VI: Poly(VPAVG)	2 gms	31.4°C	08/29/90	3 in Petri Dishes
VII: Poly(VPGVG)*	800 mg	24.8°C	9/25/90	Coacervate in γ-Irradiated Syringe
VIII: Poly(IPGVG)*	800 mg	12.9°C	9/25/90	Coacervate in γ-Irradiated Syringe
I: Poly(VPGVG)* (add'l shipment)	5 gms	25.5°C	05/09/91	6 in Sterile Tubes
IX: Poly(IPGG)	4.5 gms	22.3°C	11/19/91	8 in Sterile Tubes
X: Poly(VPGG)	4 gms	47.3°C	11/19/91	8 in Sterile Tubes
XI: Poly(APGG)	5 gms	59.9°C	11/19/91	7 in Sterile Tubes
XII: Poly[0.67(VPGG),0.33(FPGG)]	5 gms	10.5°C	11/19/91	8 in Sterile Tubes
XIII: Poly[0.9(IPGG),0.1(IPGE)]	4 gms	Figs. 5 & 6	11/19/91	8 in Sterile Tubes

*Additional shipments over and above quantities contracted.

TABLE I
(continued)

Summary of Materials Prepared and Shipped

POLYMER	QUANTITY PREPARED	T_t	DATE SHIPPED	CONFIGURATION & PACKAGING
XIV: Poly(VPGG)	2.4 gms	53.7°C	3/19/92	Four (4) Syringes
XV: Poly(VPGVG)	2.4 gms	27.7°C	3/19/92	Four (4) Syringes
XVI: Poly(VPGVG)	3.6 gms	27.7°C	6/09/92	Three (3) Syringes
XVII: Poly(GGAP)	4.3 gms	N/A**	6/09/92	Three (3) Syringes
XVIII: Poly(VPGG)	3.0 gms	27.7°C	6/09/92	One (1) Aerosol Can
XIX: Poly(VPGG)	3.0 gms	53.7°C	6/09/92	One (1) Aerosol Can
XX: Poly(GGAP)	3.0 gms	N/A	7/06/92	Syringes
XXI: Poly(GGAP)	2.5 gms	N/A	7/06/92	NAmSA Testing
XXII: Poly(GGAP)	4.8 gms	N/A	8/19/92	NAmSA Testing
XXIII: Poly(VPGG)	2.1 gms	N/A	1/22/93	NAmSA Testing

**Poly(GGAP) has a T_t above 100°C, making the determination of T_t not feasible.

IV. ANIMAL MODEL STUDIES

A series of materials was supplied to Dr. Lynne Hoban for efficacy testing in animal models of adhesion formation. The results of these studies showed the polypentapeptide to be effective in preventing postoperative adhesion formation in the contaminated peritoneal model in the rat. This is a particularly relevant model for abdominal combat casualty wounds. Reports of these results are included in Appendices G and H.

Additional animal model studies were carried by the North American Science Associates, Inc. (NAmSA®) on poly(GGAP) to assess biocompatibility, and the results are shown in Table II below.

TABLE II

Summary of Biological Test Results for Poly(GGAP)*

TEST	DESCRIPTION	TEST SYSTEM	RESULTS
(1) Ames (Mutagenicity) MG019	Determine reversion rate to wild type of histidine-dependent mutants	Salmonella typhimurium	Non-mutagenic
(2) *Cytotoxicity* MG030	Agarose overlay *determine cell death and* zone of lysis	*L-929 mouse fibroblast*	Non-toxic
(3) Systemic Toxicity TU012	Evaluate acute systemic toxicity from an I.V. or I. P. injection	Mice	Non-toxic
(4) Intracutaneous Toxicity TU013	Evaluate local dermal irritant or toxic effects by injection	Rabbit	A. 1st run demonstrated minimal reaction. B. Rerun Innocuous
(5) Systemic Antigenicity TA085	Evaluate antigenicity from I.V. or I.P. injection	Guinea Pig	Non-antigenic
(6) Sensitization (Kligman Test) TA006	Dermal sensitization potential	Guinea Pigs	Non-sensitizing
(7) Pyrogenicity TU010	Determine febrile reaction	Rabbit	Non-pyrogenic
(8) Clotting Study TA038	Whole blood clotting times	Dog	Normal
(9) Hemolysis CB037	Level of hemolysis in the blood	Rabbit blood	Non-hemolytic

* Reports from North American Science Associates, Inc. (NAmSA®).

IV. PATENT APPLICATION DEVELOPED WITH SUPPORT OF THIS CONTRACT.

This contract supported the development of new intellectual property leading most directly to a patent application on drug delivery. The history of the intellectual property development is briefly given. Telephone conversations with Dr. Taffy Williams and CDR Lee Mell relevant to Navy interest in contract research in the area of drug delivery occurred in December 1989 and January 1990. Preceding those contacts, on October 28, 1989, a consulting day of D. W. Urry for this contract, there was developed a patent kernel entitled "Bioelastic Materials as Drug Delivery Systems". Following this, two half-day meetings (taken as consulting half-days on this contract) were held at Bioelastics Research, Ltd. involving CDR L. Yaffe, Dr. A. McKee and D. W. Urry on December 18 and 19, 1989 to discuss future contract applications of the bioelastic materials. In these discussions CDR Yaffe raised the issue of nanospheres in drug delivery specifically of the 0.1 micron (μm) cut-off for particulates escaping from normal vasculature. As a result during another consulting day under this contract, December 29, 1989, a second patent kernel was developed in the area of bioelastics nanospheres for drug delivery. Subsequently, Mike Reid, the manager of the peptide chemistry laboratory for Bioelastics Research, Ltd., provided a reprint on chemical clocks. Following this information brought to light by Mike Reid, another consulting day under this contract, February 24, 1990, was utilized in part to develop a third patent kernel on the concept of preprogrammed drug delivery systems using chemical clocks to trigger release by mechanochemical coupling. The three patent kernels were then combined into a patent application entitled "Bioelastomeric Drug Delivery System", which was filed on March 27, 1990. A copy of this patent application was sent with the Progress Report for Year 1 dated October 31, 1990.

IV. PUBLICATIONS

Nine publications have been published or are in press from the support of this contract. They are listed below and included as Appendices A through I.

1. D. W. Urry, "Preprogrammed Drug Delivery Systems Using Chemical Triggers for Drug Release by Mechanochemical Coupling," Am. Chem. Soc., Div. Polym. Mater.: Sci. and Eng. **63**, 329-336, 1990.

2. D. W. Urry, "Bioelastic Materials as Chemomechanically Transducing ("Smart") Matrices for Drug Delivery," In Cosmetic and Pharmaceutical Applications of Polym. (C. G. Gebelein, T. C. Cheng, and V. C. Yang, Eds.), Plenum Press, New York, 181-192, 1991.

3. Dan W. Urry, Timothy M. Parker, Michael C. Reid, and D. Channe Gowda, "Biocompatibility of the Bioelastic Materials, Poly(GVGVP) and Its γ–irradiation Cross-linked Matrix: Summary of Generic Biological Test Results," J. Bioactive Compatible Polym. **6**, 263-282, 1991.

4. Dan W. Urry, C-H Luan, T. M. Parker, D. C. Gowda, K. U. Prasad, M. C. Reid, and A. Safavy, "Temperature of Polypeptide Inverse Temperature Transition Depends on Mean Residue Hydrophobicity," J. Am. Chem. Soc. **113**, 4346-4348, 1991.

5. Frederick J. Elsas, D. Channe Gowda and Dan W. Urry, "Synthetic Polypeptide Sleeve for Strabismus Surgery," J. Pediatr. Ophthalmol. Strabismus **29**, 284-286, 1992.

6. D. W. Urry, A. Nicol, D. C. Gowda, L. D. Hoban, A. McKee, T. Williams, D. B. Olsen and B. A. Cox , "Medical Applications of Bioelastic Materials," In Biotechnological Polymers: Medical, Pharmaceutical and Industrial Applications (Charles G. Gebelein, Ed.), Technomic Publishing Co., Inc., Atlanta, Georgia, 82-103, 1993.

7. Dan W. Urry, D. Channe Gowda, Betty A. Cox, Lynne D. Hoban, Adam McKee and Taffy Williams "Properties and Prevention of Adhesions Applications of Bioelastic Materials," Mat. Res. Soc. Symp. Proc. **292**, 1993 (in press).

8. Lynne D. Hoban, Marissa Pierce, Jerry Quance, Isaac Hayward, Adam McKee, D. Channe Gowda, Dan W. Urry and Taffy Williams, Use of Polypentapeptides of Elastin to Prevent Postoperative Adhesions: Efficacy in a Contaminated Peritoneal Model," J. Surgical Res. 1993 (in press).

9. Alastair Nicol, D. Channe Gowda, Timothy M. Parker and Dan W. Urry, "Elastomeric Polytetrapeptide Matrices: Hydrophobicity Dependence of Cell Attachment from Adhesive, $(GGIP)_n$, to Non-adhesive, $(GGAP)_n$, Even in Serum", J. Biomed. Mtrls Res., 1993 (in press).

In order to have within the body of this report a more complete listing of the substance of work accomplished, the following are the abstracts from the above nine publications.

1. **"Preprogrammed Drug Delivery Systems Using Chemical Triggers for Drug Release by Mechanochemical Coupling"**: For biodegradable drug delivery systems, whether degradation occurs by enzymatic or by salt-catalyzed hydrolytic cleavage, control of hydration becomes the key to rate of degradation. When beginning with drug-doped condensed matrices, therefore, controlling the extent and location of hydration (solvent swelling) is the key to drug release whether by diffusion, by release of entrapment or by cleavage of a drug-polymer bond.

What is desired is an adequately responsive polymer coupled to chemical clocks with a broad range of selectable half-lives that would provide the trigger for swelling. Polymers that exhibit chemically modulable inverse temperature transitions are an ideal material for such drug delivery matrices and bioelastic materials (elastomeric polypeptide biomaterials) form just such matrices.

The chemical clocks can be peptide sequences containing Asn or Gln residues which, depending on the hydrophobicity of the primary structure most proximal to these carboxamide containing residues, can exhibit half-lives in phosphate buffered saline at pH 7.5 and 37°C ranging from days to decades. The half-lives of the carboxamide to carboxylate anion reaction can be further shifted by the hydrophobicity of the remainder of the polypeptide through its folding (tertiary structure) involving hydrophobic interactions. It is the rate of formation of carboxylate anions that would control the extent of swelling and, therefore, the rate of degradation.

The rate of carboxamide/carboxylate anion conversion is the initial and most significant control step for drug release but additional control steps are possible such as introducing esters into the polypeptide backbone. The proximity of the backbone ester to hydrophobic moieties will affect its rate of hydrolytic cleavage further opening access to the eroding surface. Also, the drug may be covalently attached to the polymer and the rate of this bond cleavage could also affect the rate of drug release. Of course, the drug could simply be trapped within the matrix with release occurring by diffusion on swelling and/or on backbone degradation.

Whether drug release is by controlled swelling with enhanced rate of degradation or by contraction with expelling of contents, the devices can be designed with site specificity based on a unique chemical aspect of the target site much as proteins themselves are brought to fold or unfold, to assemble or disassemble and to function with highly cooperative response profiles and to do so at specific sites.

As stated by Pitt and Schindler, the ultimate objective is "to develop delivery systems which respond according to the biochemical need for the drug, comparable to the way the human body exercises control over releases and distribution of its own endogenous biological agents" (Pitt, C.G. and Schindler, A., in Progress in Contraceptive Delivery Systems (E. Hafez & W. Van Os, Eds.), MTP Press Ltd. 1, 17-46, 1980). To the extent that proteins control release and distribution of endogenous agents and to the extent that protein structure is modulated and function is achieved by the mechanism considered here for drug delivery, that is, chemical modulation of inverse temperature transitions or hydration mediated apolar-polar interaction free energies, then the approach outlined here provides a significant step toward that ultimate objective.

2. **"Bioelastic Materials as Chemomechanically Transducing ("Smart") Matrices for Drug Delivery"**: For biodegradable drug delivery systems, control of hydration can be a key to rate of degradation. When beginning with drug-doped condensed matrices, controlling the rate of hydration (swelling) can then become the key to drug release. One approach is to use responsive polymers coupled to chemical clocks with selectable half-lives. Polymers that exhibit chemically modulable inverse temperature transitions are an ideal material for such drug delivery matrices and elastomeric polypeptides form such matrices. The chemical clocks can be peptide sequences containing Asn or Gln residues (or other suitably unstable chemical groupings) which, depending on the hydrophobicity of

adjacent residues, can exhibit half-lives from days (or less) to decades. The half-lives of the [CONH2→COO^-] reaction can be further shifted by hydrophobic tertiary structure. It is the rate of carboxylate anion or other polar species formation which would then control the extent of swelling and rate of degradation.

Whether drug release is by controlled swelling with enhanced rate of degradation, or by contraction with expelling of contents, the devices can be designed with site specificity based on a unique chemical aspect of the target site such as proteins themselves are brought to fold or unfold, to assemble or disassemble and to function at specific sites with highly cooperative chemical response profiles. To the extent that protein structure is modulated and function is achieved by the mechanism considered here for drug delivery, that is, chemical modulation of the temperature of inverse temperature transitions, then the approach outlined here provides a step toward desired biological specificity with the capacity to release the drug being coupled to the unique chemistry of the diseased state. Such a bioresponsive drug delivery system would constitute a particular integration of the diagnostic-therapeutic pair in which a protein-based polymer is sumultaneously the sensor of the need for, and the deliverer of, the drug and wherein the spent device can be degraded with release of simple amino acids and the chemical sensor.

3. **"Biocompatibility of the Bioelastic Materials, Poly(GVGVP) and Its γ–irradiation Cross-linked Matrix: Summary of Generic Biological Test Results"**: The complete series of the recommended generic biological tests for materials and devices in contact with tissues and tissue fluids and blood have been carried out by an independent testing laboratory on the elastic protein-based (bioelastic) polymer, poly(L-Val^1-L-Pro^2-Gly^3-L-Val^4-Gly^5) with a degree of polymerization greater than 120, and its 20 Mrad γ-irradiation cross-linked elastic matrix, X^{20}-poly(VPGVG). The specific tests and the summarized results given in parentheses are: (1) the Ames mutagenicity test (non mutagenic), (2) cytotoxicity-agarose overlay (non-toxic), (3) acute systemic toxicity (non-toxic), (4) intracutaneous toxicity (non-toxic), (5) muscle implantation (favorable), (6) acute intraperitoneal toxicity (non-toxic), (7) systemic antigenicity (non-antigenic), (8) dermal sensitization – the Magnusson and Kligman maximization method (non-sensitizing), (9) pyrogenicity (non-pyrogenic), (10) Lee White clotting study (normal clotting time), and (11) *in vitro* hemolysis test (non-hemolytic). Thus, this new elastomeric polypeptide biomaterial which is based on the most striking repeating sequence in the mammalian elastic fiber exhibits an extraordinary biocompatibility. This parent bioelastic material and a wide range of component peptide variations are under development for an equally wide range of potential medical applications such as prevention of adhesions, drug delivery, and synthetic arteries.

4. **"Temperature of Polypeptide Inverse Temperature Transition Depends on Mean Residue Hydrophobicity"**: This publication is a communication in the Journal of the American Chemical Society. It relates to the N00014-89-C-0282 contract in that this contract contributed polymers for the hydrophobicity scale and in that a completed hydrophobicity scale is necessary for the proper desig~ of matrices for the prevention of adhesions. This is particularly the case as it has been shown (see Figures

1, 2 and 3 of this report and abstract #9 below) that cell adhesion in the presence of serum is proportional to hydrophobicity.

5. **"Synthetic Polypeptide Sleeve for Strabismus Surgery"**: A synthetic polypentapeptide sleeve was placed around the superior rectus muscle of five New Zealand white rabbits in hopes of preventing postoperative fibrous scarring. Two forms of the polypentapeptide were used. No significant inflammation or scarring occurred with either form of the polypentapeptide when compared to controls. One form elicited a fibrous membrane surrounding the sleeve within 2 weeks. The other elicited no such reaction after 2 months. The latter form of the polypeptapeptide may be useful in preventing scarring following strabismus surgery.

6. **"Medical Applications of Bioelastic Materials"**: Repeating peptide sequences that occur in mammalian elastic fibers exhibit interesting properties of entropic (ideal) elasticity and of folding and assembling on raising the temperature. When correctly understood, these properties make it possible to design and synthesize elastomeric polypeptides capable of exhibiting additional properties and functions with both medical and non-medical applications.

For medical applications, it is first necessary to demonstrate biocompatibility. This has been done for the parent elastic protein-based polymer, poly(Val^1-Pro^2-Gly^3-Val^4-Gly^5) or poly(VPGVG), which exhibits an extraordinary biocompatibility as the polymer and as the γ-irradiation cross-linked elastomeric matrix, designated as X^{20}-poly(VPGVG). As cells do not adhere to this matrix and as no fibrous capsule forms around it when implanted, the matrix and other states of the material have potential for use in the prevention of post-operative, post-trauma adhesions. Specific studies underway include a contaminated peritoneal model using the rat, a strabismus surgery model using the rabbit eye, and just beginning a total artificial heart model as a bridge to transplantation using the calf.

Because cell attachment sequences can be introduced into the matrix which result in cell adhesion, spreading and growth to confluence, such modified elastomeric matrices become candidates for use in a wide range of possible tissue reconstructions. In general, the concept is to design a functional scaffolding for a particular tissue into which the appropriate cells can migrate and within which they function and remodel the prosthetic material into a natural tissue with slow degradation and removal of the temporary synthetic scaffolding.

These compliant, biocompatible matrices also have significant potential for use in drug delivery. This is made especially attractive because, in addition to being able to introduce chemical clocks that would control their rate of degradation as desired, they can be designed to swell or to contract in response to a relevant chemical signal which may be associated with a particular diseased state.

This capacity to design the matrices to contract and relax in response to a chemical signal (i.e., to function in chemomechanical transduction) and even to carry out more diverse free energy transductions

known to be important in living organisms suggests further dynamic roles in their potential medical applications.

7. **"Properties and Prevention of Adhesions Application of Bioelastic Materials"**: The origins, syntheses, variable composition and physical properties of bioelastic materials are discussed. The latter includes their capacity to undergo inverse temperature transitions to increased order on raising the temperature and to be designable to interconvert free energies involving the intensive variables of mechanical force, temperature, pressure, chemical potential, electrochemical potential and light.

Bioelastic materials include analogues and other chemical variations of the viscoelastic polypeptide, poly(Val-Pro-Gly-Val-Gly), and cross-linked elastomeric matrices thereof. This parent material has been shown to be remarkably biocompatible; it can be minimally modified to vary the rate of hydrolytic breakdown; it can contain enzymatically reactive sites; and it can have cell attachment sites included which promote excellent cell adhesion, spreading and growth to confluence.

One specific application is in the prevention of postoperative adhesion. There are some 30,000,000 surgical procedures per year in this country and a large portion of these would benefit if a suitable material were available for preventing adhesions. Bioelastic materials have been tested in a contaminated peritoneal model, and promising preliminary studies have been carried out in the rabbit eye model for strabismus surgery. In the peritoneal model, 90% of the 29 control animals exhibited significant adhesions; whereas, only 20% of the 29 animals using gas sterilized matrices had significant adhesions. On the basis of this data, it appears that cross-linked poly(VPGVG) is an effective physical barrier to adhesion formation in a trauma model with resulting hemorrhage and contamination.

The potential use of bioelastic materials as a pericardial substitute following the more than 400,000 open heart surgeries per year in the U.S. is under development beginning with the use of bioelastic matrices to prevent adhesions to the total artificial heart being used as a bridge to heart transplantation such that the site will be less compromised when receiving the donor heart.

8. **"Use of Polypentapeptides of Elastin to Prevent Postoperative Adhesions: Efficacy in a Contaminated Peritoneal Model"**: We investigated the use of a sheet of polypentapeptide of elastin as a physical barrier to adhesion formation in a contaminated peritoneal wound model. The material can be supplied in sheets, liquid or foam. A total of 88 rats were studied with random assignment of animals to three study groups: Control (29), polypentapeptide steam sterilized (30), and polypentapeptide gas sterilized (29). Animals were anesthetized and a laparotomy conducted to reveal the cranial portion of the ileum. The abdominal wall muscle peritoneum was excoriated until hemorrhage was noted. In sham animals, there was no physical barrier placed between bowel loop and the abdominal wall. In the study groups, the polypentapeptide sheet was placed directly over the excoriated area. The intestinal loop was then loosely secured to excoriated area with 2-0 nylon (stay suture) which was tied subcutaneously in all groups. Four puncture wounds were made with a 20-gauge hypodermic needle in the bowel that was apposed to the excoriated periteneal musculature which allowed leakage of intestinal contents and

contamination. On day 7 post surgery, the animals were anestetized and the stay suture was removed. On day 14, all animals were sacrificed and adhesions graded. The incidence of significant adhesions was 28% for barrier group versus 90% for control animals ($p<0.05$). The results of this study indicate that the polypentapeptide of elastin sheet is an effective physical barrier in this surgically induced contaminated wound model. Further investigation utilizing liquid and foam forms of this material are needed to completely evaluate the potential applications.

9. **"Elastomeric Polytetrapeptide Matrices: Hydrophobicity Dependence of Cell Attachment from Adhesive, $(GGIP)_n$, to Non-adhesive, $(GGAP)_n$, Even in Serum"**: The cross-linked polytetrapeptide matrices based on the repeating amino acid sequences, GGAP, GGVP and GGIP, were prepared and tested for cell adhesion promoting activity in both the absence and presence of fetal bovine serum. For comparison, X^{20}-poly(GVGVP), a matrix previously shown to be a poor support for cell attachment and spreading, was included. In the absence of serum, all three polytetrapeptide-based matrices and the polypentapeptide-based matrix were negative for the adhesion of fibroblasts and endothelial cells. In the presence of serum, various sub-maximal levels of cell adhesion were found for all matrices except for the matrix based on GGAP. An apparent correlation was noted between the degree of cell attachment to the different polytetrapeptide-based matrices and the hydrophobicity of those matrices where increased hydrophobicity results in increased cell attachment. The property of being refractory to ligamentum nuchae fibroblast and human umbilical vein endothelial cell adhesion in the presence of serum indicates a potential use for X^{20}-poly(GGAP) in the development of, for example, additional physical barriers for the prevention of post-surgical and post-trauma adhesions.

VI. SYNERGISM OF EFFORTS IN THIS CONTRACT WITH THOSE OF N000-90-C-0265 ENTITLED, "DEVELOPMENT OF BIOELASTIC MATERIALS FOR ASPECTS OF WOUND REPAIR."

In contract No. N00014-90-C-0265 among other efforts there was to be designed a vascular sleeve which will biodegrade in several months time; there was developed an aerosol can packaging of solutions in order to spray a unique bioelastic material into a gaping wound as a foam which was to harden at body temperature to form a temporary cast which would be readily removed on lowering the temperature to just below ambient temperatures, and there was support for materials testing in commercial laboratories as required for FDA approval.

The questions of rate of degradation were relevant to the present contract in that the sheets are to degrade after repair is complete where as Drs. L. Hoban and T. Williams of the NMRI Combat Casualty Care Unit have found that sheets of poly(VPGVG) placed intraperitoneally were still present as such after 90 days *in situ*. The efforts in No. N00014-90-C-0265 will provide the information required to determine ways of increasing rate of degradation. An attractive possibility as a form of the bioelastic material is to produce a foam. As this will be done under No. N00014-90-C-0265, this form of the

material will have been developed and could be tested under this contract (N00014-89-C-0282) for use in the prevention of adhesions. Finally before an Investigational Device Exemption (IDE) can be filed with the FDA, biocompatibility must be established. Accordingly, some eight tests on poly(GGAP) were carried out (see Table II, page 10) in an independent accredited laboratory, namely North American Sciences Association (NAmSA®). A portion of the money to support this testing was in No. N00014-90-C-0265, but inasmuch as the results were relevant to many medical applications and specifically to the prevention of adhesions application, i.e. to this contract, monies from this contract (N00014-89-C-0282) were utilized to proportionately cover the cost of testing.

VIII. CONCLUSIONS

This contract for the development of bioelastic materials for the prevention of adhesions has been extraordinarily successful. That success is apparent in the substance of the publications (9) and patent application that have resulted. Initially it was necessary to establish the biocompatibility of the basic material X^{20}-poly(VPGVG). This elastic matrix was found to exhibit exceptional biocompatibility, being essentially ignored by the host. Directly to the objective, in a study which used 88 animals the material has been found to prevent post-surgical, post-trauma adhesions in the rat contaminated peritoneal model, it also appears to be effective in preventing adhesions in a strabismus surgery model in the rabbit eye, and is presently being tested for preventing adhesions in the use of the total artificial heart as a bridge to transplantation with the longer range goal of developing an artificial pericardium for use in the 400,000 cardiopulmonary bypass operations per year in the U.S.

There are now two steps to take in proceeding to develop the material as a commercial product for the prevention of adhesions. One is to introduce chemical clocks that will provide for a slow removal of the matrix, and the second is to develop a bioproduction method that will allow for a lower cost for the bioelastic material. With a bioproduction of the bioelastic polymers, the biocompatibility and efficacy studies will be repeated, an Investigational Device Exemption (IDE) is to be sought which would constitute approval to proceed with clinical trials. With the much less expensive bioproduction, e.g. by *E. coli* fermentation, being expected to reduce the cost of preparation by a factor of 100, it is difficult to justify the expenditure of tens of millions of dollars for clinical trials being dependent on the more expensive chemical preparation. Work is proceeding to accomplish these two steps, and they can now occur based on the above successful demonstration of biocompatibility and efficacy for the prevention of adhesions in the contaminated periotoneal model which is most directly relevant to abdominal combat casualty wounds.

PREPROGRAMMED DRUG DELIVERY SYSTEMS USING CHEMICAL TRIGGERS FOR DRUG RELEASE BY MECHANOCHEMICAL COUPLING. Dan W. Urry, Bioelastics Research, Ltd., 1075 13th Street, South, Birmingham, AL 35205, and Laboratory of Molecular Biophysics, School of Medicine, University of Alabama at Birmingham, UAB Station, P. O. Box 300, Birmingham, AL 35294.

INTRODUCTION:

Sequential polypeptides such as poly(Val[1]-Pro[2]-Gly[3]-Val[4]-Gly[5]) and poly(Val[1]-Pro[2]-Gly[3]-Gly[4]) and numerous analogs when combined with water form viscoelastic phases which when cross-linked result in soft, compliant, elastomeric matrices (1-3). The polypentapeptide has been shown to be biocompatible both before and after cross-linking (4). As implants, they are biodegradable leading to the release of products natural to the body. As such, these materials, referred to as elastomeric polypeptide biomaterials or simply bioelastic materials, are obvious candidates for drug delivery systems. In general, they may be used as matrices to be doped with drugs for diffusional release. They may be prepared with widely different water compositions, with a wide range of hydrophobicities, with almost any desired shape and porosity, and with a variable degree of cross-linking. Accordingly, these matrices are candidates for holding a significant variety of drugs for diffusional release. Furthermore, residues with functional side chains, such as Glu, Ser, Lys, etc., can be introduced in order to attach drugs covalently. Then drug release could also be dependent on the rate of cleavage of the drug-polymer bond. These are quite straight-forward utilizations of bioelastic materials as drug delivery systems for which here is provided the initial statement. There are, however, additional capacities that these materials have which provide for site specificity and a special degree of control over the time release profile This involves the capacity of these materials to be designed to function as free energy transducers, for example, specifically to exhibit thermomechanical transduction, chemomechanical transduction (mechanochemical coupling) and reasonably electromechanical transduction (5). Associated with these transduction processes can be dramatic local swelling in aqueous media. Volume changes of an order of magnitude and more can be triggered with the expected consequence of a greatly enhanced rate of degradation in the biological milieu. There are chemical clocks, for example, for triggering chemomechanical transduction with the potential of half-lives varying one-thousand fold from days to decades. An understanding of this process as it relates to drug delivery systems will be conveyed in what follows. Basically, it involves chemical modulation of inverse temperature transitions to bring about disassembly and unfolding of polymer matrices or to bring about a contraction that effects release, and the specific example used will be elastomeric polypeptide matrices

INVERSE TEMPERATURE TRANSITIONS AND THERMOMECHANICAL TRANSDUCTION:

The phenomena of inverse temperature transitions in aqueous systems is general and involves amphiphilic systems, commonly polymers, with the proper balance and arrangement of apolar and polar moieties. The polar species contribute to the solubility in water at low temperature, a solubility that results in waters of hydrophobic hydration for the apolar moieties. The waters of hydrophobic hydration, often referred to as clathrate or clathrate-like water, have most interesting thermodynamic properties of an exothermic heat of hydration (a negative ΔH) and a negative entropy of hydration (6,7). On raising the temperature, by means of an endothermic transition (8), the low entropy waters of hydrophobic hydration become bulk water with a significant increase in solvent entropy as the polymers fold and aggregate optimizing intra- and intermolecular contacts between hydrophobic (apolar) moieties with a somewhat lesser decrease in polymer entropy than increase in solvent entropy. Such polymers when their transitions occur between 0° and 100°C can be of interest in the aqueous milieu that occurs in biology.

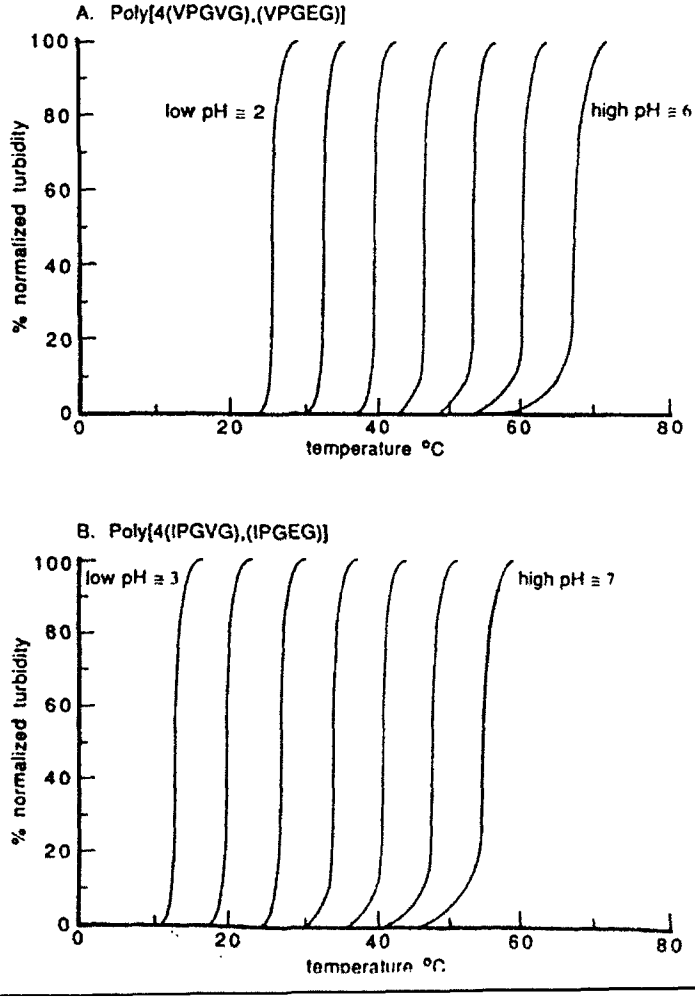
The polypentapeptide, poly(Val[1]-Pro[2]-Gly[3]-Val[4]-Gly[5]) or poly(VPGVG), is a particularly well-balanced polymer for biological interest as its transition is just complete near 37°C. Below 25°C, it is miscible with water in all proportions where it exhibits a secondary structural feature called a β-turn in which there occur hydrogen bonds between the Val[1] C-O and the Val[4]-NH moieties (9). On raising the temperature, the polypentapeptide folds into a loose helix in which the dominant interturn hydrophobic contacts involve the Val[1]γCH_3 moieties in one turn and the Pro[2]-βCH_2 moiety in the adjacent turn (10). The loose helical structure is called a dynamic β-spiral and is proposed to be the basis for the entropic elastomeric force exhibited by this material once cross-linked (11). Concomitant with the folding is an assembly of β-spirals to form a twisted filament which optimizes intermolecular hydrophobic contacts.

When poly(VPGVG) is cross-linked , for example, by 20 Mrads of γ-irradiation, an elastomeric matrix is formed which is swollen below 25°C but which on raising the temperature through the transition contracts with the extrusion of sufficient water to decrease the volume to one-tenth and to decrease the length of a strip of matrix to 45% of its swollen length (2). This thermally driven contraction can be used to lift weights that are one thousand times the dry weight of the matrix. This is thermomechanical transduction. As will be discussed below, any chemical means of reversibly or irreversibly shifting the temperature of the transition can be used, isothermally, to achieve chemomechanical transduction or mechanochemical coupling.

CHEMICAL MODULATION OF INVERSE TEMPERATURE TRANSITIONS AND CHEMOMECHANICAL TRANSDUCTION

The temperature of inverse temperature transitions can be changed by changing the hydrophobicity of the polymer. For example, make the polypeptide more hydrophobic, as with poly(Ile^1-Pro^2-Gly^3-Val^4-Gly^5) where replacing Val^1 by Ile^1 represents the addition of one CH_2 moiety per pentamer, and the temperature of the transition decreases by 20°C from 30°C for poly(VPGVG) to 10°C for poly(IPGVG) (1). Similarly, decreasing the hydrophobicity as by replacing Val^4 by Ala^4, i.e., removing two CH_2 moieties per pentamer, and the temperature of the transition is raised by some 40°C to 70°C. Now thinking in terms of a generalized hydrophobicity scale, the COOH moiety may be considered more hydrophobic than the COO^- moiety such that by simply changing the pH the temperature of the transition can be changed. The transition temperature can be lowered by decreasing the pH and raised by increasing the pH. If an intermediate temperature is maintained, then a 20 Mrad cross-linked matrix of poly[4(VPGVG),(VPGEG)] where E = Glu will contract on lowering the pH and relax or swell on raising the pH (12). The temperature of the transition in phosphate buffered saline will shift some 50°C from about 20°C at low pH, giving COOH, to nearly 70°C at neutral pH where all of the carboxyls have been converted to carboxylate anions. For similarly cross-linked poly[4(IPGVG),(IPGEG)], the temperature of the inverse temperature transition shifts from near 10°C for COOH to over 50°C for COO^- (5). This is shown schematically in Figure 1A and B. Interestingly, for the more hydrophobic 4% Glu polypentapeptide, it takes twice as many carboxylate anions to shift the transition to 40°C as for the less hydrophobic polypentapeptide.

FIGURE 1:



While at the isothermal condition of 37°C, on lowering the pH, that is, on raising the chemical potential, μ, of proton, these matrices can exert forces, f, sufficient to lift weights that are a thousand times their dry weight. This is chemomechanical transduction, also called, mechanochemical coupling. The mechanism whereby this occurs is called an hydration mediated apolar-polar repulsion free energy and is characterized by the statement $(\partial\mu/\partial f)_n < 0$, that is, the change in chemical potential with respect to force at constant matrix composition is a negative quantity (13). Such matrices take up protons on stretching, i.e., stretching exposes more hydrophobic groups to water which makes the COO^- moieties energetically less favored. This is quite distinct from the previously considered charge-charge repulsion mechanism for mechanochemical coupling where $(\partial\mu/\partial f)_n > 0$ and where stretching of such matrices causes the release of protons. The new mechanism appears to be an order of magnitude more efficient in converting chemical work into mechanical work such that it can be expected that proteins in carrying out their diverse functions would utilize the mechanism requiring the least amount of chemical metabolic energy.

It may be emphasized here that any chemical means of changing the mean hydrophobicity of the polymer, such as an acid/base titrable function, dephosphorylation/phosphorylation, reduction/oxidation of a redox couple, etc., can be used to bring about contraction/relaxation. It is anticipated that proteins function utilizing these processes.

In noting the general relevance of the above statements, it should be appreciated that chemically induced structural (conformational) changes are examples of mechanochemical coupling, i.e., of chemomechanical transduction. Accordingly, chemically induced swelling of drug loaded matrices to achieve drug release is an example of mechanochemical coupling just as chemically induced contraction to achieve drug release from within a contracting envelope would be mechanochemical coupling (See below). And it can be appreciated that polymer matrices (whether polypeptide or other polymers) capable of chemically modulable inverse temperature transitions would be functioning much as proteins function.

CHEMICAL CLOCKS FOR CONTROLLING RATES OF DRUG RELEASE

It is possible to have polymer matrices that are so dense and hydrophobic as to render them essentially non-biodegradable. This is even possible with polypeptide matrices as has been shown with poly(Glu-co-Leu), where a 1:1 copolymer was recovered intact after 15 months *in vivo* (14). Nonetheless, treatment with dilute NaOH and neutralizing to pH 7 resulted in complete biodegradation. By the mechanism described above where $(\partial\mu/\partial f)_n < 0$, the pKa of a carboxyl moiety can be increased by increasing vicinal hydrophobicity (13,15). Presumably, the dilute base treatment was sufficient to result in the formation of interfacial COO^- moieties which could start the swelling process required for degradation. What is required is a chemically modulable polymer system as part of which there could be a controlled rate of presentation of more polar species such as the carboxylate anion.

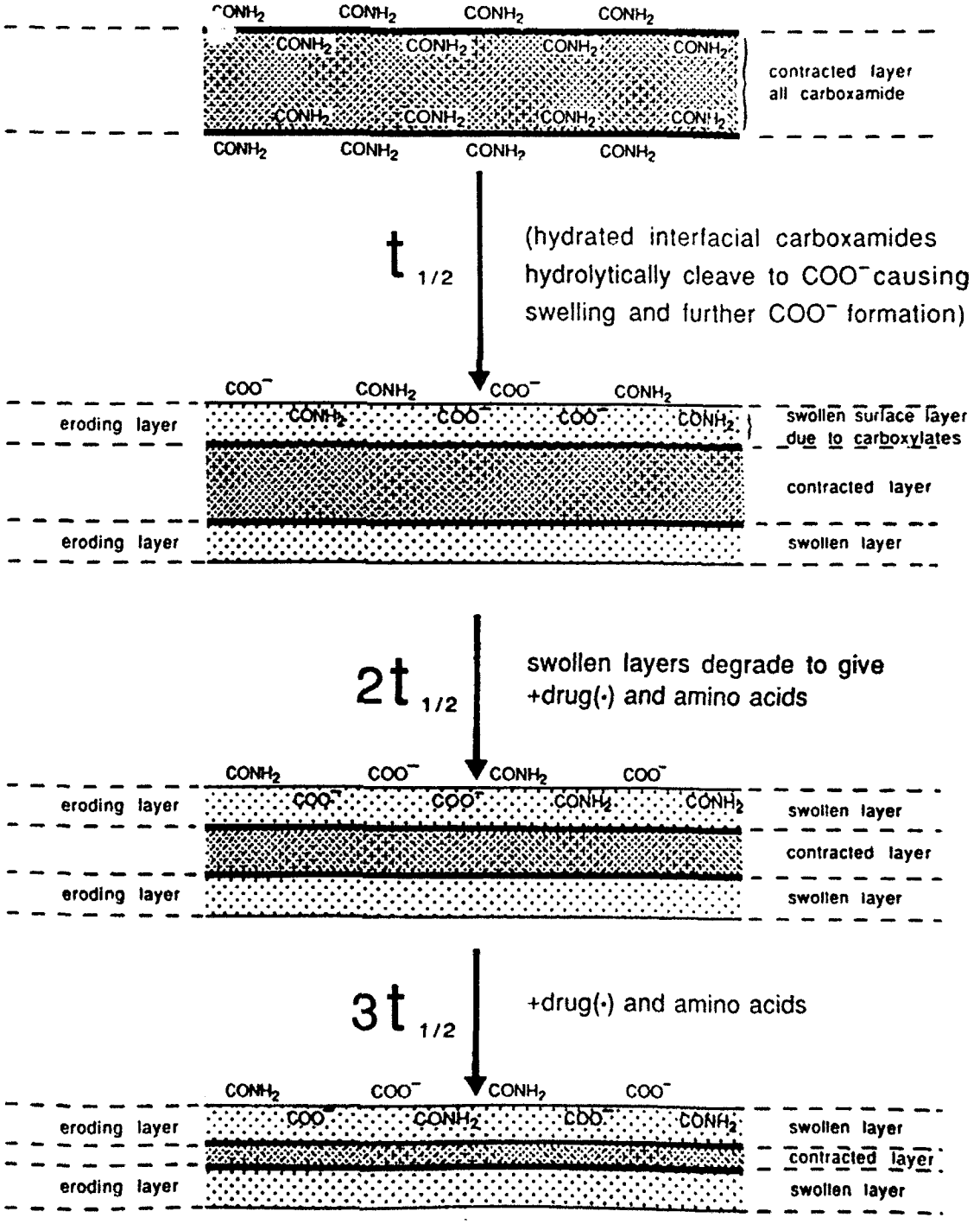
Asparagine (Asn) and glutamine (Gln) residues can be the desired chemical clocks. Robinson has pointed out with more than sixty pentameric sequences in which the central residue was Asn or Gln that, at 37°C in phosphate buffered saline pH 7.5 and ionic strength 0.15, the half-lives of the carboxamide side chains could be varied from six days for Gly-Ser-Asn-His-Gly to 3409 days for Gly-Thr-Gln-Ala-Gly with Gly-Ile-Asn-Ala-Gly having an intermediate half-life of 507 days (16). More hydrophobic residues could be expected to slow the decay further, while not necessarily water soluble as the free pentamers, the more hydrophobic carboxamide containing pentamers could be part of larger polypeptides. While a range for the half-lives of a factor of 500 was demonstrated, with greater hydrophobicity in the primary structure and with polypeptide folding (tertiary structure) also contributing, one could expect to vary the half-times from days or fraction of days to decades.

With the already defined sequential polypeptide matrices capable of exhibiting inverse temperature transitions, such as poly(VPGVG), poly(IPGVG), poly(VPAVG), poly(VPGG), poly(VAPGVG), poly(VPGFGVGAG), etc., where A = Ala and F = Phe with water contents ranging from less than 10% to greater than 90% (1,17-19), it is proposed that the rate of degradation of such matrices *in vivo* could be varied from exhibiting half-lives of days to decades. The scenario would be one of interfacial hydrolytic cleavage of a carboxamide to carboxylate anion with resultant local swelling leading to polymer degradation. The trigger of interfacial carboxamide hydrolytic cleavage can be preprogrammed by sequence, and by more general polypeptide hydrophobicity, to occur at a given rate. This would be a key control step with the consequence of local swelling. The second control step would be the rate of degradation of the polypeptide involved in the local

swelling which could also be enhanced by having esters in the backbone (i.e., depsipeptides). The rate of hydrolytic cleavage of the backbone ester would depend on the hydrophobicity of residues in the sequence most proximal to the ester residue. A third control step could be having the drug bound to the polypeptide through side chains by bonds that would also have different rates of cleavage. Thus, preprogramming of drug release, by release from entrapment, by controlled swelling and subsequent degradation and/or by subsequent hydrolytic cleavage of the polymer-drug bond, becomes possible when using matrices which undergo chemically modulable inverse temperature transitions.

DRUG DELIVERY USING MATERIALS CAPABLE OF MECHANOCHEMICAL COUPLING: SELECTED EXAMPLES:

FIGURE 2: Carboxamide Chemical Clock for Controlling Rate of Surface Swelling Followed by Degradation of Monolith Slab, Uniformly Doped with Drug



Given the number of ways that contraction and relaxation (swelling) can be chemically achieved, it is apparent that there are innumerable delivery constructs that may be conceived. Here, however, only three will be explicitly noted. They are: (1) monoliths with preprogrammed chemical clocks where drug release is brought about by chemically triggered relaxation (swelling), (2) mechanochemical pumps where drug release is achieved by chemically driven contraction as in the wringing out of a sponge, and (3) nanospheres that may be site-targeted by size and also by chemical triggering. In the latter two examples, site-targeting could be achieved by a relatively small difference in a chemical property such as pH. This can occur due to hydrophobicity induced pK shifts and the cooperativity of the charging process involved in mechanochemical coupling (13,15).

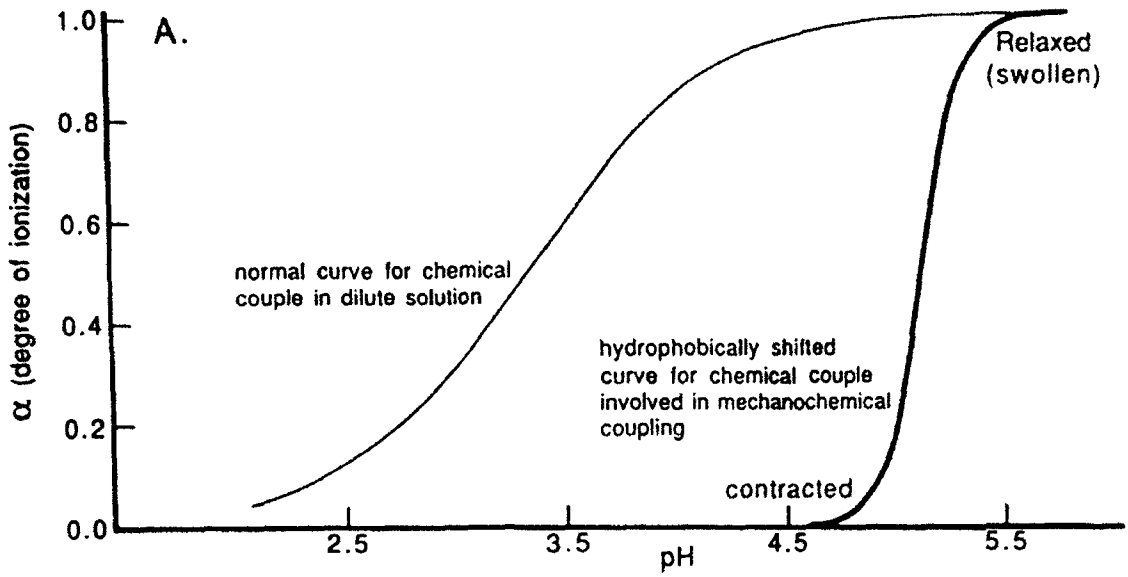
Carboxamide Chemical Clocks for Controlling Rate of Surface Swelling Followed by Degradation of Doped Monolith: This is considered by preparing an otherwise non-degradable matrix containing a uniform distribution of drug. Degradation of a slab monolith can occur once sufficient hydration is achieved, and sufficient hydration of the relatively hydrophobic matrix can occur once the temperature of the inverse temperature transition is raised above 37°C by a chemical perturbation. The rate limiting chemical perturbation is the rate at which spontaneous hydrolysis occurs at the aqueous milieu-slab interface. An example of the chemical trigger can be asparagine (Asn) or glutamine (Gln) within the polymer sequence and the rate at which the carboxamide $\Rightarrow$ carboxylate anion chemical perturbation occurs can be controlled by the hydrophobicity of adjacent residues and the folding of the remainder of the polymer chain. By properly choosing the adjacent residues and the hydrophobicity of the matrix in general, the half-life for the hydrolysis can be altered from days to decades. As depicted in Figure 2 and demonstrated in Figure 1, once a carboxylate anion is formed, it shifts the temperature for the swelling transition for the chains, within some few tens of Ångstroms of the ion, from below to above body temperature. This will cause a surface layer to swell at a rate dependent upon the $CONH_2 \Rightarrow COO^-$ transition. As the surface layer swells, the drug can be released either by diffusion or if covalently bonded to the polymer, by subsequent polymer backbone cleavage and/or polymer-drug bond cleavage. The rates of these latter cleavages could be sufficiently slow as to contribute to the overall rate of drug release or they could be faster than the $CONH_2 \Rightarrow COO^-$ transition such that the deamidation step would be rate limiting. As the surface layer swells and sufficient water and salts can approach new carboxamides, they can also undergo cleavage with their characteristic rates. The process continues until the slab is degraded.

It should also be appreciated that the chemical clock could be the drug-polymer bond itself (for example, an ester or amide bond to a Glu, Asp, or Lys residue) within the matrix with an appropriately altered vicinal hydrophobicity to control rate of hydrolytic or enzymatic cleavage.

Hydrophobicity Induced pKa Shifts and Cooperativity for Ionization in Matrices Capable of Mechanochemical Coupling: At the molecular level, the mechanism for mechanochemical coupling is considered in water- and structure-limited systems to arise out of the competition of the apolar (hydrophobic) and polar species for hydration. As the hydrophobic moiety with its waters of hydration becomes too proximal to a polar species with its hydration shell, the interceding water molecules cannot simultaneously provide for the free energies of hydration of both the apolar and polar species with the result of an unfavorable increase in free energy. This has profound effects on an ionizable polar species such as the $COOH/COO^-$ couple. With the cross-linked matrix of poly[4(VPGVG),(VPGEG)], at low pH the matrix is contracted. Even though the relatively hydrophobic matrix when contracted is more than 50% water by weight, the system is water-limited. As the pH is raised, the ionization of the COOH is delayed because of inadequate waters of hydration for the COO^- moiety. This leads to an increase in the pKa in proportion to the hydrophobicity of the matrix (13,15). As the first few COO^- moieties do appear, they cause the matrix to swell making it easier for subsequent anions to form. There is therefore a cooperative effect, and the titration curve becomes steeper as shown in Figure 3A. If the titrable group is a cationic chemical couple as $-NH_3^+/NH_2$ or as the His^+/His (imidazolium/imidazole) couple, the free energy of the more polar species is again most significantly raised. The result is a pKa that is lowered and again a cooperative effect is observed as depicted in Figure 3B. The cooperative effect, apparent as the steepened pH dependence for the degree of ionization and for the mechanochemical coupling, means that a site with a more acidic pH could either selectively cause a contraction (for an anionic chemical couple) or a swelling (for a cationic chemical couple) of a matrix capable of exhibiting chemical modulation of an inverse temperature transition.

FIGURE 3: Cooperativity and pK shifts in Sufficiently Hydrophobic Matrices Capable of Mechanochemical Coupling

A. Anionic Chemical Couple (e.g. $COOH/COO^-$)



B. Cationic Chemical Couple (e.g. imidazolium/imidazole, His^+/His)

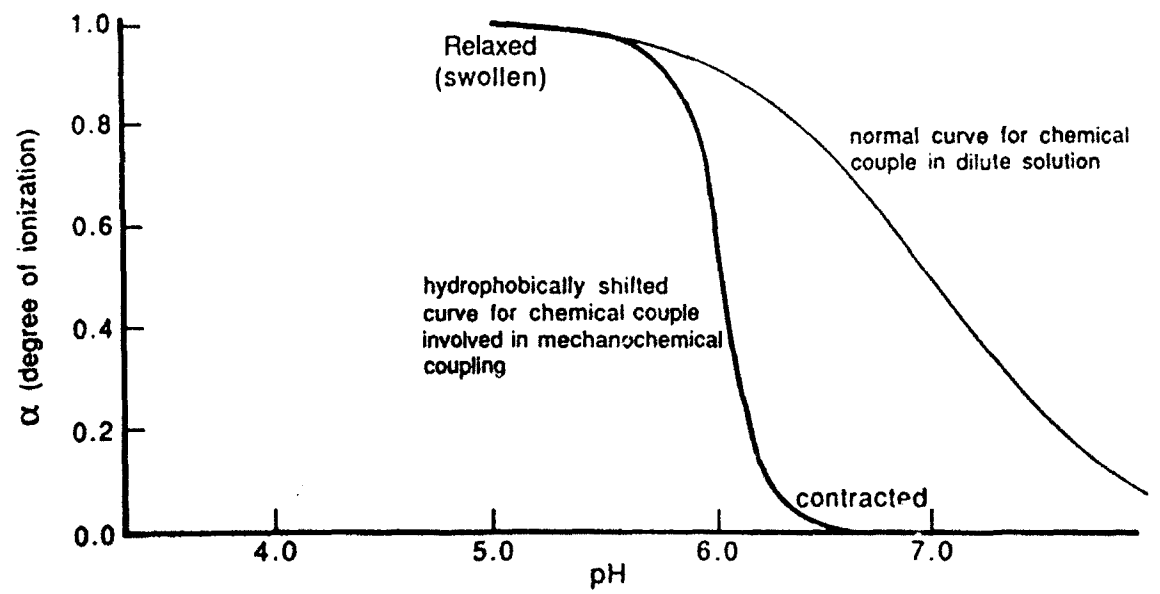
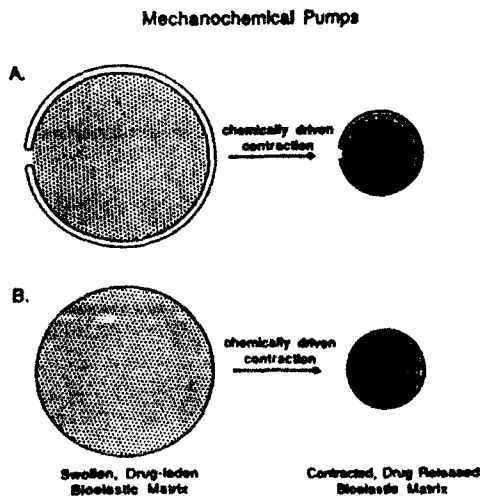


FIGURE 4:



Mechanochemical Pumps: Two constructs for mechanochemical pumps may be considered, one where the mechanochemical matrix surrounds the drug as shown in Figure 4A and the other a monolith which on contraction much as a sponge delivers its drug (Figure 4B). Both of these constructs could provide site specific release of drug, whenever a site had a sufficiently distinct chemical property. A unique property of a site could be a pH other than the usual extracellular pH; it could be an increased enzyme activity; it could be the increase concentration of either component of a redox couple; it could be an enhanced oxidative capacity with excess superoxide, hydrogen peroxide or hypochlorite, etc.; or it could even be a different salt concentration (20). Also, rather than a contractile release, a degradational release could be achieved by a site specific chemical signal resulting in a swelling and enhanced degradational release as considered for the chemical clock except that the site would provide the stimulus rather than the preprogrammed chemical clocks.

Nanospheres: In the study of the nucleation and accretion that occurs just preceding the inverse temperature transition, it has been seen that particles of different radii can be produced. At this stage, diameters from 10 to 1000 nm have been observed (21,22). Such particles could be cross-linked by any of a number of ways including γ-irradiation, and they could have drugs attached. Accordingly, sites differentially reachable by particle size could be sites for selective drug delivery, and size specificity could readily be coupled to chemical specificity as noted above.

CONCLUSION:

For biodegradable drug delivery systems, whether degradation occurs by enzymatic or by salt-catalyzed hydrolytic cleavage, control of hydration becomes the key to rate of degradation. When beginning with drug-doped condensed matrices, therefore, controlling the extent and location of hydration (solvent swelling) is the key to drug release whether by diffusion, by release of entrapment or by cleavage of a drug-polymer bond.

What is desired is an adequately responsive polymer coupled to chemical clocks with a broad range of selectable half-lives that would provide the trigger for swelling. Polymers that exhibit chemically modulable inverse temperature transitions are an ideal material for such drug delivery matrices and bioelastic materials (elastomeric polypeptide biomaterials) form just such matrices.

The chemical clocks can be peptide sequences containing Asn or Gln residues which, depending on the hydrophobicity of the primary structure most proximal to these carboxamide containing residues, can exhibit half-lives in phosphate buffered saline at pH 7.5 and 37°C ranging from days to decades. The half-lives of the carboxamide to carboxylate anion reaction can be further shifted by the hydrophobicity of the remainder of the polypeptide through its folding (tertiary structure) involving hydrophobic interactions. It is the rate of formation of carboxylate anions that would control the extent of swelling and, therefore, the rate of degradation.

The rate of carboxamide/carboxylate anion conversion is the initial and most significant control step for drug release but additional control steps are possible such as introducing esters into the polypeptide backbone. The proximity of the backbone ester to hydrophobic moieties will affect its rate of hydrolytic cleavage further opening access to the eroding surface. Also, the drug may be covalently attached to the polymer and the rate of this bond cleavage could also affect the rate of drug release. Of course, the drug could simply be trapped within the matrix with release occurring by diffusion on swelling and/or on backbone degradation.

Whether drug release is by controlled swelling with enhanced rate of degradation or by contraction with expelling of contents, the devices can be designed with site specificity based on a unique chemical aspect of the target site much as proteins themselves are brought to fold or unfold, to assemble or disassemble and to function with highly cooperative response profiles and to do so at specific sites.

As stated by Pitt and Schindler, the ultimate objective is "to develop delivery systems which respond according to the biochemical need for the drug, comparable to the way the human body exercises control over releases and distribution of its own endogenous biological agents" (23). To the extent that proteins control release and distribution of endogenous agents and to the extent that protein structure is modulated and function is achieved by the mechanism considered here for drug delivery, that is, chemical modulation of inverse temperature transitions or hydration mediated apolar-polar interaction free energies, then the approach outlined here provides a significant step toward that ultimate objective.

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APPENDIX B

BIOELASTIC MATERIALS AS CHEMOMECHANICALLY TRANSDUCING ("SMART") MATRICES FOR DRUG DELIVERY

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For biodegradable drug delivery systems, control of hydration can be a key to rate of degradation. When beginning with drug-doped condensed matrices, controlling the rate of hydration (swelling) can then become the key to drug release. One approach is to use responsive polymers coupled to chemical clocks with selectable half-lives. Polymers that exhibit chemically modulable inverse temperature transitions are an ideal material for such drug delivery matrices and elastometric polypeptides form such matrices. The chemical clocks can be peptide sequences containing Asn or Gln residues (or other suitably unstable chemical groupings) which, depending on the hydrophobicity of adjacent residues, can exhibit half-lives from days (or less) to decades. The half-lives of the [$CONH_2 \rightarrow COO^-$] reaction can be further shifted by hydrophobic tertiary structure. It is the rate of carboxylate anion or other the polar species formation which would then control the extent of swelling and rate of degradation.

Whether drug release is by controlled swelling with enhanced rate of degradation, or by contraction with expelling of contents, the devices can be designed with site specificity based on a unique chemical aspect of the target site much as proteins themselves are brought to fold or unfold, to assemble or disassemble and to function at specific sites with highly cooperative chemical response profiles. To the extent that protein structure is modulated and function is achieved by the mechanism considered here for drug delivery, that is, chemical modulation of the temperature of inverse temperature transitions, then the approach outlined here provides a step toward desired biological specificity with the capacity to release the drug being coupled to the unique chemistry of the diseased state. Such a bioresponsive drug delivery system would constitute a particular integration of the diagnostic-therapeutic pair in which a protein-based polymer is simultaneously the sensor of the need for, and the deliverer of, the drug and wherein the spent device can be degraded with release of simple amino acids and the chemical sensor.

Cosmetic and Pharmaceutical Applications of Polymers
Edited by C.G. Gebelein *et al.*, Plenum Press, New York, 1991

INTRODUCTION

This manuscript conceptually addresses the potential, as drug delivery matrices, of a relatively new class of biomaterials within the general category of what has recently become termed protein-based polymers.* The specific protein-based polymers of interest here are described as elastometric polypeptide biomaterials or bioelastic materials which may be designed to exhibit efficient chemomechanical transduction.

PROTEIN-BASED POLYMERS

A protein-based polymer is a chain molecule comprised of repeating peptide sequences where the repeating unit may be as small as a few residues or as large as hundreds of residues. The repeating unit could be as small as a repeating dipeptide, as in the poly(L,D)dipeptide of gramicidin A which forms ion selective transmembrane channels, or it could be a repeating domain of hundreds of residues as a model for the repeating domains of transmembrane pumps and channels and of multi-subunit enzymes. Protein-based polymers may be designed as simplified models for developing an understanding of the driving forces involved in protein function. And once the driving forces of protein function are adequately understood, protein-based polymers may be designed to perform many of the highly specific and cooperative functions of complex natural proteins. Protein-based polymers can then be developed as drug delivery systems not simply as inert carriers of drugs but rather as dynamic target specific deliverers of pharmaceuticals. Proteins are chemomechanical transducers in that a chemical signal (a chemical concentration change resulting for example in ligand binding) induces a conformational change (a mechanical response) resulting in function. This is most readily visualized in chemically-driven contraction and relaxation; and indeed protein-based polymers have been designed to exhibit chemically-driven contraction and relaxation, i.e. chemomechanical transduction.[1-5]

BIOELASTIC MATERIALS AS TRANSDUCING MATRICES

For purposes of the following, the repeating peptide sequence of primary interest is Val^1-Pro^2-Gly^3-Val^4-Gly^5 which, as a protein-based polymer, is written poly(VPGVG). When γ-irradiation crosslinked by 20 Mrads, this protein-based polymer forms an elastomeric matrix designated as X^{20}-poly(VPGVG).[1,2] This matrix exhibits mechanothermal coupling by means of an inverse temperature transition, that is, at low temperature the matrix is swollen in water and on raising the temperature the matrix contracts, decreasing volume by an order of magnitude and length by a factor of greater than 2. This is thermomechanical transduction.[3] The matrix can be modified such that, when it is more hydrophobic (apolar), the transition occurs at a lower temperature and, when it is more hydrophilic (polar), the transition occurs at a higher temperature.

The change in hydrophobicity or hydrophilicity can be achieved in many ways. The addition of a functional group, that can be in either a more or less polar state, can be used to change the hydrophobicity and,

*The first symposium on protein-based polymers occurred as part of the April 23-29, 1990 National American Chemical Society meeting and was organized by D. Kaplan, M. Marron, and D. Tirrell who developed the term.

thereby, to change the temperature of the transition. A convenient functional group is the carboxyl group, which may either be in the less polar (more hydrophobic) [COOH] state, or the more polar (less hydrophobic) [COO−] state. Such a functional moiety is available in the glutamic acid residue, abbreviated Glu or E. In the elastomeric matrix, X^{20}-poly-[4(VPGVG)(VPGEG)], there are four Glu residues per 100 residues of polypentapeptide. At low pH, about 2, essentially all of the side chains of the glutamic acid residues are protonated giving only [COOH] moieties. In this case, in phosphate buffered saline [(0.15 N NaCl and 0.01M phosphate)], the transition is centered near 25°C and the matrix contracts on raising the temperature from 20° to 30°C as shown in Figure 1.[4] At pH 4.5, when there are about two carboxylate anions [COO−] per 100 residues, the contraction occurs on raising the temperature from 45° to 60°C (see Figure 1).

The capacity to change the temperature at which thermomechanical transduction occurs by changing the pH means that it is possible to remain at an intermediate temperature, say 37°C, and change the pH to achieve contraction and relaxation. At low proton concentration (high pH), the matrix is swollen. On increasing the proton concentration, i.e., on lowering the pH and protonating the carboxylate anions, the matrix contracts to less than one-half the swollen length and can lift weights in doing so that are a thousand times the matrix dry weight. The process is reversible such that the cycle can be repeated many times. This is chemomechanical transduction or mechanochemical coupling.[4]

1. Cooperativity in the Contraction/Relaxation Transition

A most interesting feature about this pH driven contraction/relaxation is shown in Figure 2.[5] When the elastomeric matrix is under load, the pH dependence of the contraction and of the degree of ionization α of the carboxyl moiety is very steep. In this case, 75% of the ionization, i.e., some 75% of the transition, is achieved in a ΔpH of 0.5, whereas, a

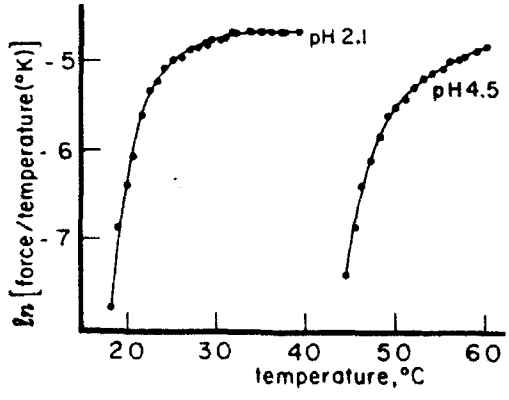


Figure 1. Thermomechanical transduction exhibited by X^{20}-poly[4(VPGVG),(VPGEG)]. At pH 2.1 contraction begins on raising the temperature from just below 20°C and is completed just above 30°C. At pH 4.5 contraction does not begin until above 40°C and is completed just above 60°C. (Reproduced with permission from reference 4).

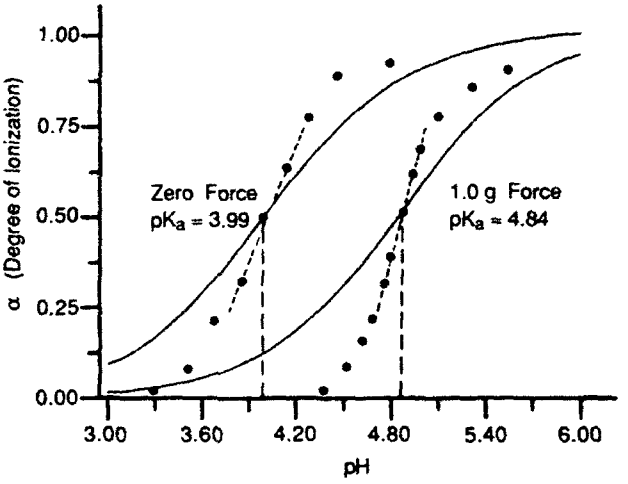


Figure 2. When X^{20}-poly[4(VPGVG),(VPGEG)] is titrated without force being applied to the elastomer, an acid base titration curve is obtained with a pK_a of 3.99. After the elastomer has been stretched by the application of a force, the pK_a is raised, in this study, to 4.84 and the steepness of the curve is increased. See text for discussion. (Reproduced with permission from reference 5).

ΔpH of 2.5 is required to achieve the same change in the degree of ionization for a standard titration curve (see the solid lines of Figure 2). The steepness of the curve is a function of the hydrophobicity of the matrix, and it is the exposure of hydrophobic groups to the aqueous medium on stretching (applying a force) that causes the increase in steepness and the shift in pK_a of Figure 2. Thus, the hydrophobicity of the matrix is one means of controlling the pK_a,[6] and of controlling the steepness of the transition.[5] The steepened transition is described as a positive cooperativity.

Cooperativity is a property of multisubunit enzymes and other multisubunit proteins which can be quantified from the steepness of the transition by means of a Monod-type analysis.[7,8] Using this analysis of protein functionality, the change in slope on stretching with exposure of more hydrophobic groups gives a change in Gibb's free energy per mole of ligand. With the ligand being the [H$^+$] moiety, analysis of the data in Figure 2 gives a value of about 2RT (S.Q. Peng and D. W. Urry, unpublished results). This is the same energy as determined from the shift in pK_a on applying the force, i.e., $\Delta G/\Delta n = \Delta\mu = -2.3RT\Delta pK_a \approx 2RT$. Thus cooperativity, one of the more exquisite properties of protein function, is apparent in this protein-based polymer designed to exhibit chemomechanical transduction, i.e., chemically-induced contraction/relaxation.

The process of contraction or of relaxation (swelling) can be the controlling element for drug release. Accordingly, a drug delivery device could be designed to be responsive to a small change in pH and to a pH, though distinctly different, that is not greatly displaced from normal physiological pH.

2. Efficiency in the Conversion of Chemical Energy into Mechanical Work

An engine is "a machine designed for the conversion of energy into useful mechanical motion".[9] The contractile bioelastic matrices for drug delivery are engines of a type that convert chemical energy ($\Delta\mu\cdot\Delta n$) into the useful mechanical motion of drug delivery. Here $\Delta\mu$ is the change in chemical potential which may be written as $-2.3RT\Delta pH$ in Gibb's free energy/mole, when the chemical species is the proton and Δn is the change in number of moles of protonated species in the protein-based polymer, both for the amount of work, w, achieved. The efficiency, η, of such an engine can be given by $w/\Delta\mu\cdot\Delta n)$, where the work can be measured as a force, f, working through a distance, Δl, as for example in the lifting of a weight.[10]

Now it is of interest to compare the efficiency of the bioelastic matrix, X[20]-poly[4(VPGVG),(VPGEG)], with that of crosslinked poly(methacrylic acid), $[(-CH_2CCOOH-CH_2-)_n]$.[10-12] The latter utilizes a charge-charge repulsion mechanism to extend the chain, wherein the chains become fully extended at 50 to 60% [COO$^-$]. On lowering the charge-charge repulsion by protonation, the extended chains collapse and achieve a contraction to about one-half length on lowering [COO$^-$] to 0 to 10%. Thus, to achieve the contraction of shortening to about one-half the length with the capacity to lift weights of about a thousand times the dry weight of the matrix requires the Δn due to changing some 40[COO$^-$] moieties to [COOH] per 200 backbone atoms. To achieve this, the ΔpH needs to be about 2.5[13] giving 3.5kcal/mole. This is summarized in Table 1, along with the comparable values for X[20]-[4(VPGVG),(VPGEG)], which uses a mechanism referred to as apolar-polar repulsive hydration free energies. This protein-based engine is capable of doing a similar amount of work in the contraction cycle, i.e., to contract to about half its length, and to pick up weights that are one thousand times its dry weight. But in this

Table 1. Comparison of Chemomechanical Transduction Efficiencies

$$\eta = w/(\Delta\mu\cdot\Delta n)$$

Where w = work = $f\Delta l$; $\Delta\mu$ = change in chemical potential; Δn = change in moles of polymer species; $\Delta\mu\cdot\Delta n$ = the chemical energy.

$\eta_{c,c}$ = efficiency of Charge-Charge repulsion mechanism
(as exemplified by polymethacrylic acid)

w: $\Delta f \approx 1/2$; f ≈ 1000 x dry weight
Δn: 4(COO$^-$→COOH) per 300 backbone atoms
$\Delta\mu$: ($\Delta\alpha$ of ≈ 0.6→$\Delta pH \approx 2.5$) ≡ 3.5 kcal/mole

$\eta_{a,p}$ = efficiency of Apolar-Polar repulsion free energy mechanism
(as exemplified by X[20]-poly[4(VPGVG),(VPGEG)])

w: $\Delta f \approx 1/2$; f ≈ 1000 x dry weight
Δn: 4(COO$^-$→COOH) per 300 backbone atoms
$\Delta\mu$: ($\Delta\alpha$ of ≈ 0.8→$\Delta pH \approx 0.5$) ≡ 0.7 kcal/mole

RESULT:

$$\eta_{a,p}/\eta_{c,c} > 10$$

case, only 4 [COO⁻] moieties are converted to [COOH] per 300 backbone atoms and the ΔpH required to do so can be as small as 0.5[5] such that the chemical energy, $\Delta\mu \cdot \Delta n$, required is more than an order of magnitude smaller. The contractile bioelastic matrices can achieve ten times the amount of mechanical work for a given change in chemical concentration. These materials would therefore, be much more sensitive (10x) in responding to the altered chemical composition of a diseased state and in delivering a drug.

BIOELASTIC MATERIALS AND THE CONCEPT OF THE DIAGNOSTIC-THERAPEUTIC PAIR

For some time there has been an objective of integrating into a single drug delivery device both the diagnostic and therapeutic steps. The device would be a bioresponsive drug delivery system which, for example in cases of Type I diabetes, would have the capability of measuring the blood glucose level and in response would provide release of the appropriate dose of insulin.[14]

As noted above, bioelastic materials can be designed to contract or to relax in response to a chemical signal and, thereby, could be utilized to achieve drug delivery in response to a changed chemical environment.[15,16] Chemically driven contraction and relaxation has been demonstrated by a change in pH. Theoretically, chemomechanical transduction could be achieved by any functional moiety attached to the contractile protein-based polymer that could occur in both a less polar (more hydrophobic) state and a more polar (less hydrophobic) state. This would include the imidazole moiety of the histidine residue, in which case the effect of a ΔpH would be just the reverse of the glutamic acid residue, that is , for the histidine residue, relaxation would occur on lowering the pH and contraction would occur on raising the pH to physiological levels and presumably the pK_a can be shifted by shifting the hydrophobicity of the protein-based polymer. This is depicted in Figure 3. The possible functional moieties could also include chemical groupings that could be oxidized and reduced as in many of the prosthetic groups which occur in enzymes. In this regard, it has been proposed that the enzymes that achieve function on changing the redox state of prosthetic groups (e.g., of the hemes of cytochromes, of the nicotinamide adenine and flavine adenine dinucleotides, etc.) do so by the change in polarity of the prosthetic group causing a change in the folded state of the protein wherein changing the folded state achieves function. The change in redox state shifts the temperature of an inverse temperature transition to a temperature above or below physiological temperature to result in a differently folded state as demonstrated in Figure 1, using pH for X^{20}-poly-[4(VPGVG),(VPGEG)]. With the appropriate redox potential for a redox couple in the contractile matrix, the chemomechanical transduction could be coupled to the oxidative state of an enzyme that is sensitive to the chemical to be modulated. For example, for glucose, there is an oxygenase and a dehydrogenase that use the dinucleotide prosthetic groups noted above.

The potential of the bioelastic materials is that the elastomeric matrix itself both could be the sensor of the altered chemical situation of the diseased state and, as an integral part of the sensing process, could be deliverer of the therapeutic dose of the drug required to correct or improve the diseased condition. Furthermore, the properly designed bioelastic matrix has the potential of doing so with the sensitivity, specificity and cooperativity that one has come to appreciate of enzymes and other proteins. In addition the protein-based polymer as a drug delivery device would degrade, once completing its task, with release of

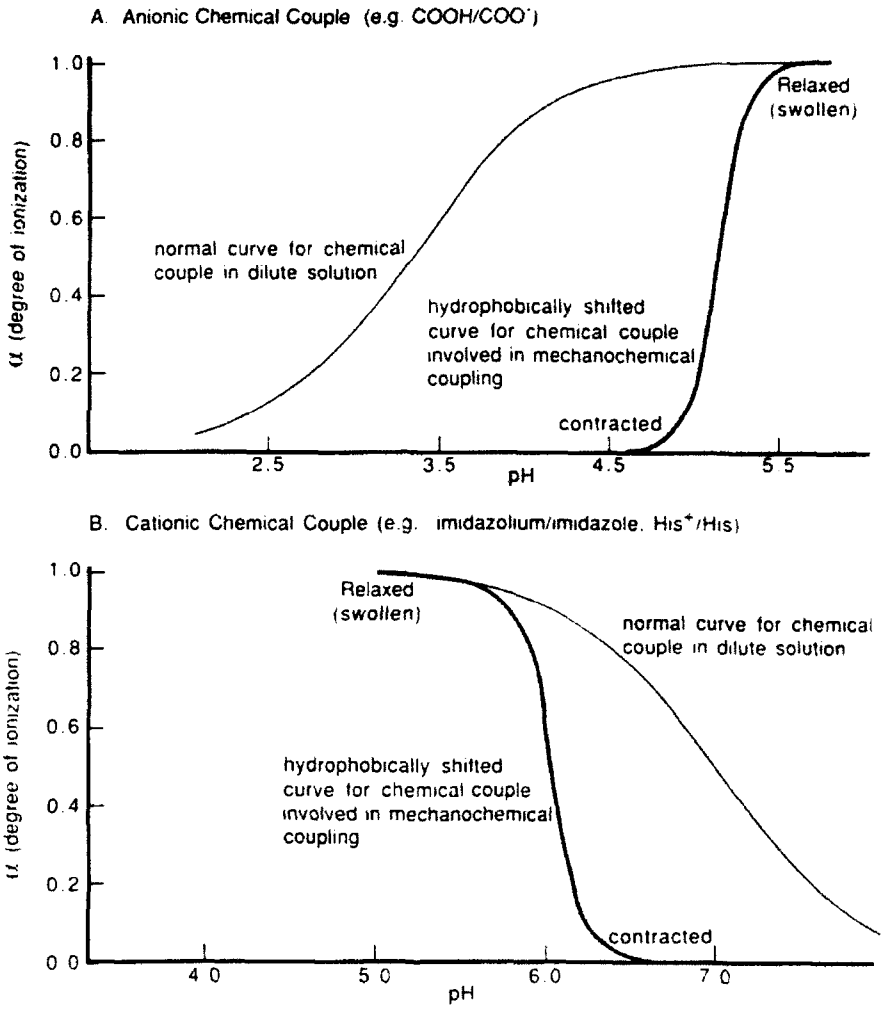


Figure 3. Schematic representations in pK_a and increases in cooperativity on increasing hydrophobicity for [A], an anionic chemical couple and [B], a cationic chemical couple. (Reproduced with permission from reference 15).

amino acids and of what most commonly could be a physiological chemical sensing moiety when the sensing element is not an amino acid with a functional side chain. The downside of using protein-based polymers in this way is that they could elicit an antigenic or an immunogenic (foreign body) type of response. Such a possibility does not seem to have deterred the consideration of proteinuceous nanoparticles, i.e., gelatin, serum albumin, etc.,[17] which do not exhibit the transducing properties central to the bioelastic materials, and the primary bioelastic material, X^{20}-poly(VPGVG), studied thus far appears to be quite innocuous and benign particularly in comparison to Dacron® and Dexon®.[18]

In a series of ten tests required for devices and materials in contact with tissue, tissue fluid, and blood, the bioelastic material, poly-(VPGVG) and its γ-irradiation crosslinked matrix, has been found by an independent testing laboratory to be biocompatible.

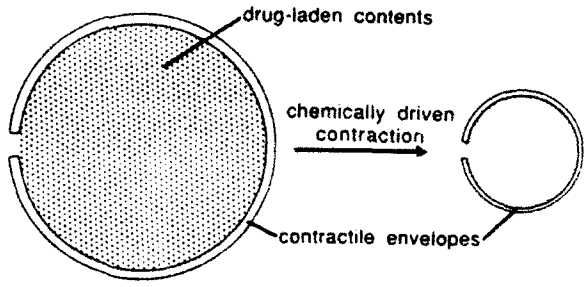


Figure 4. Mechanochemical pump having a contractile envelope and drug-laden contents. (Reproduced with permission from reference 15).

EXAMPLES OF BIOELASTIC CONSTRUCTS AS "SMART" MATRICES FRO DRUG DELIVERY

The following are examples of three distinct bioelastic constructs which utilize the chemomechanical transducing (mechanochemical coupling) properties of the bioelastic materials. As they have been described previously,[15,16] they will only be briefly treated here. These may be compared to the innovative uses of polymeric materials as drug delivery systems recently reviewed by Langer.[19,20]

1. Mechanochemical Pumps

A conceptually simple construct is to have a contractile envelope surrounding a core filled with drug. The envelope can be chemically induced to contract expelling its contents. This is depicted in Figure 4.

2. Transducing Nanoparticles

By appropriate use of concentration, temperature and time, it is possible to form aggregates of the bioelastic materials with radii of controllable sizes ranging from 50 to 500 nm[21,22] (see Figure 5), which could be fixed by crosslinking at the desired size. This allows use of the differing sizes for the permeability barriers to particles of endothelium and basement membrane vasculature that occur normally and in diseased states. Thus a contracted nanoparticle could be sized to pass through the vasculature in order to approach the site of the diseased tissue better.[23] The contracted nanoparticle would be drug-laden and with the proper chemical sensing element could be induced to swell and release the drug in the chemical environment of the diseased state (see Figure 6).

3. Transducing Monoliths with Preprogrammed Rates

An interesting example of using chemomechanically transducing bio-

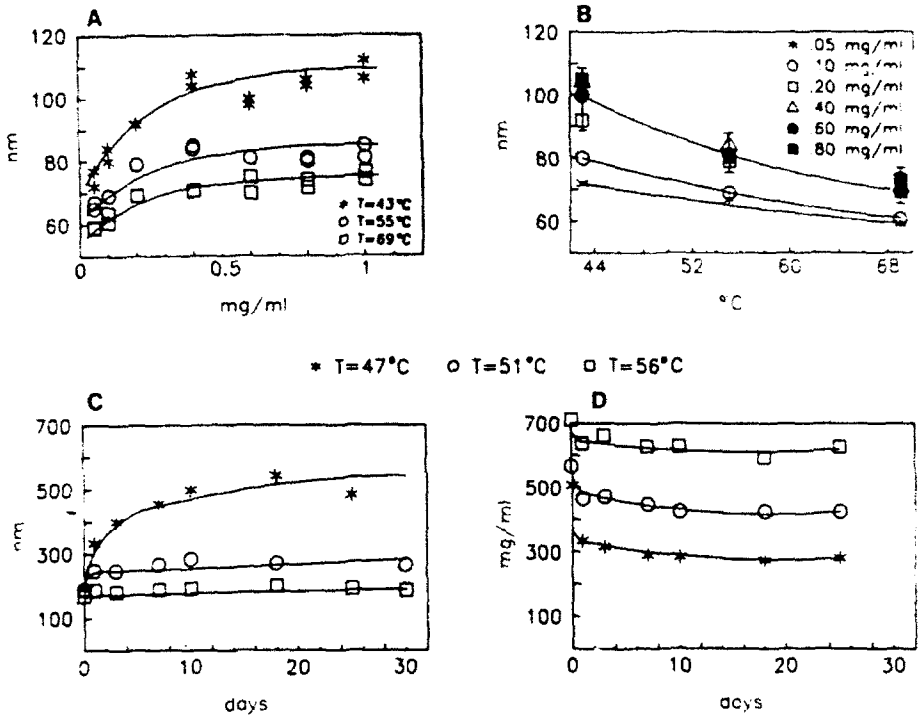
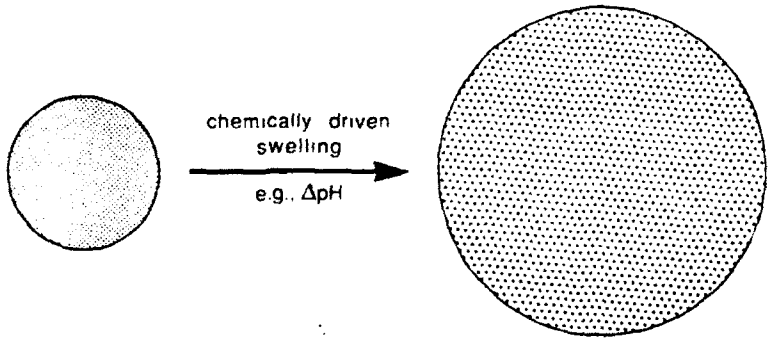


Figure 5. Concentration, temperature and time dependence for nanoparticles formed from poly(VPGVG). Recognizing that the ordinates are given as radii, particle diameters from 100 to 1000 nm are observed. (Reproduced from reference 21 with permission).



Diagnostic–Therapeutic Pair

1. Device uses altered (diseased) chemical environment to bring about drug release
2. Spent swollen device degrades releasing
 a Peptides and amino acids – e.g., (Val. Pro. Gly)
 b Sensor – e.g., (His. Glu. Prosthetic Group)

Figure 6. Protein-based nanospheres for drug delivery. The example is given wherein a contracted nanoparticle containing trapped drug is chemically induced to swell releasing its load of drug. (Adapted with permission from reference 15).

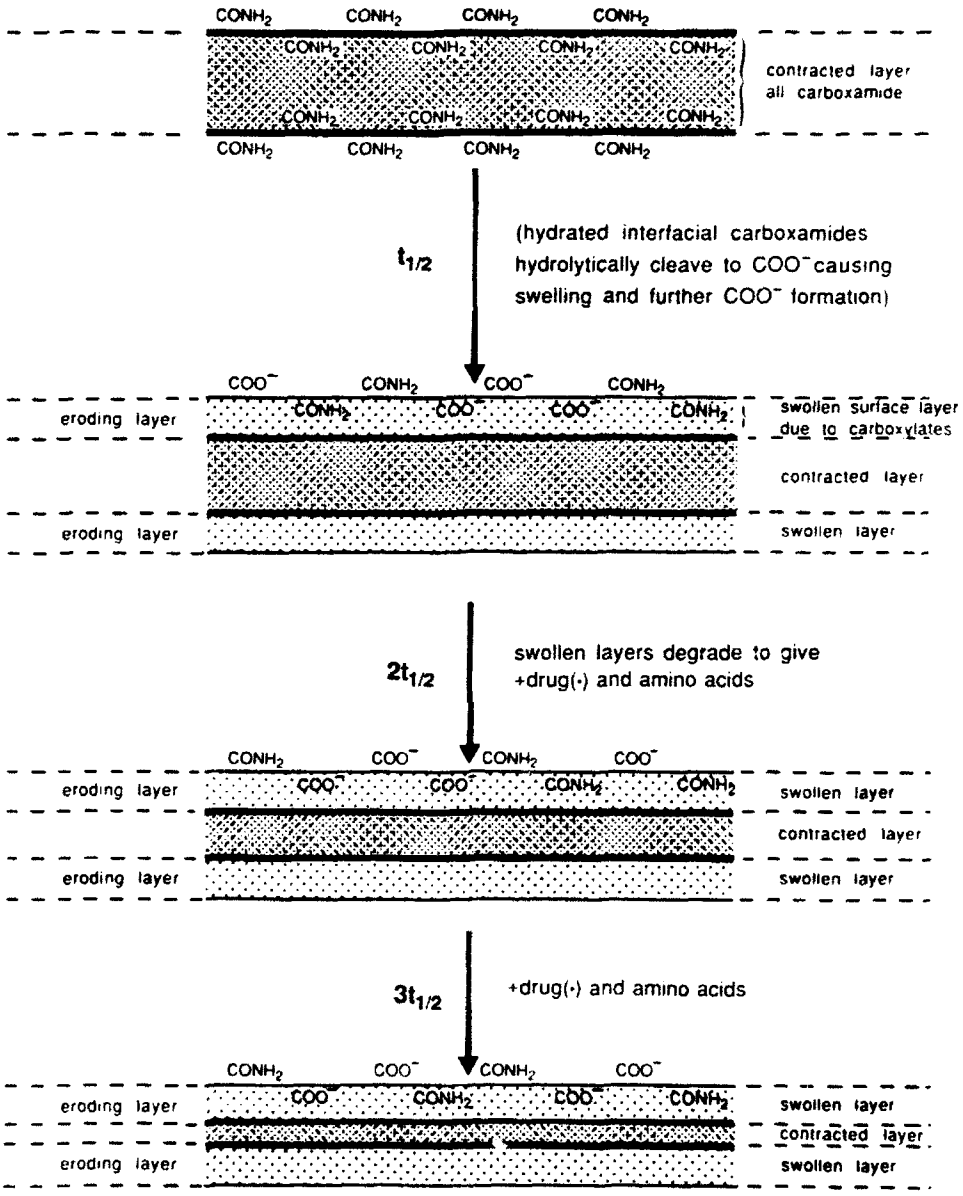


Figure 7. A monolith slab uniformly doped with trapped drug molecules, which contains as an example carboxamide [$CONH_2$] chemical clocks which, when at the surface, are spontaneously hydrolytically cleaved with predeterminable half-lifes, $t_{1/2}$, to give carboxylate anions [COO^-] which, in turn, cause the surface layer to swell releasing the trapped drug molecules. (Reproduced with permission from reference 15).

elastic matrices can be demonstrated by using a chemically labile moiety which, when it breaks down with a characteristic half-life, causes the matrix to swell and release its contents. For example, the amino acid residues, asparagine and glutamine, contain the carboxamide moiety [$CONH_2$] which spontaneously hydrolyzes in phosphate buffer at physiological pH to form the carboxylate anion [COO^-]. The formation of the carboxylate anion, as in the pH titration of Figure 2 to produce the [COO^-] moiety, raises the temperature of the inverse temperature transition to above physiological temperatures with the result of the local matrix-swelling and allowing release of drug. This is depicted in Figure 7. In addition, there could be any other chemically labile protective groups such as those commonly used in peptide chemistry. Also, of course, unstable linkages could be placed in the polypeptide backbone. An ester would be a structurally compatible unstable backbone linkage, but indeed, any chemically unstable spacer could be introduced into the chain of the protein-based polymer provided that it did not destroy the transducing properties and, of course, the drug could be covalently attached to the polymer chain by means of a linkage with the desired rate of cleavage.

ACKNOWLEDGMENT

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APPENDIX C

Biocompatibility of the Bioelastic Materials, Poly(GVGVP) and Its γ-Irradiation Cross-Linked Matrix: Summary of Generic Biological Test Results

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ABSTRACT: The complete series of the recommended generic biological tests for materials and devices in contact with tissues and tissue fluids and blood have been carried out by an independent testing laboratory on the elastic protein-based (bioelastic) polymer, poly(L-Val^1-L-Pro^2-Gly^3-L-Val^4-Gly^5) with a degree of polymerization greater than 120, and its 20 Mrad γ-irradiation cross-linked elastic matrix, X^{20}-poly(VPGVG). The specific tests and the summarized results given in parentheses are: (1) the Ames mutagenicity test (non-mutagenic), (2) cytotoxicity-agarose overlay (non-toxic), (3) acute systemic toxicity (non-toxic), (4) intracutaneous toxicity (non-toxic), (5) muscle implantation (favorable), (6) acute intraperitoneal toxicity (non-toxic), (7) systemic antigenicity (non-antigenic), (8) dermal sensitization—the Magnusson and Kligman maximization method (non-sensitizing), (9) pyrogenicity (non-pyrogenic), (10) Lee White clotting study (normal clotting time), and (11) *in vitro* hemolysis test

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(non-hemolytic). Thus, this new elastomeric polypeptide biomaterial which is based on the most striking repeating sequence in the mammalian elastic fiber exhibits an extraordinary biocompatibility. This parent bioelastic material and a wide range of component peptide variations are under development for an equally wide range of potential medical applications such as prevention of adhesions, drug delivery, and synthetic arteries.

INTRODUCTION

The American Society for the Testing of Materials (ASTM) under the guidance of Committee F-4 on Medical and Surgical Materials and Devices published in 1987 the Standard Practice for Selecting Generic Biological Test Methods for Materials and Devices (ASTM Designation F748-87). For implanted materials and devices in contact with tissue and tissue fluids, nine tests were considered applicable and for implanted devices in contact with blood, another two tests were listed as applicable. This report summarizes the results of eleven tests** carried out by the test facility, North American Science Associates, Inc. (NAmSA®), on the first of a new class of biomaterials referred to as elastomeric polypeptides or bioelastic materials. The material is also called an elastic protein-based polymer in that the initial polymer derives from a repeating pentamer sequence that occurs in mammalian elastic fibers [1,2]. The polypentapeptide is poly(Val^1-Pro^2-Gly^3-Val^4-Gly^5) which may be abbreviated as poly(VPGVG) or as any of the permutations, e.g., poly(GVGVP) wherein it is the pentamer GVGVP that is activated and polymerized. With degrees of polymerization of between 100 and 200 the end two or three residues can generally be considered to be inconsequential so that any convenient permutation may be used in the synthesis. For secondary structural reasons of a hydrogen bond between the Val^1CO and the Val^4 NH, the polypentapeptide is most commonly referred to as poly(VPGVG).

Poly(VPGVG) and numerous analogs have been characterized by a large number of physical and computational methods [3–10]. Poly(VPGVG) is soluble in water in all proportions below 25°C, but on raising the temperature to 37°C it undergoes a transition in which the polypentapeptide folds into a more ordered state with formation of a viscoelastic condensed phase that is about 40% peptide, 60% water by weight. On γ-irradiation cross-linking of the viscoelastic phase (called

**The nine applicable tests include a carcinogenicity test which, when the mutagenicity test is sufficiently favorable as in the present case, is not considered necessary. Thus carcinogenicity has not been included and an antigenicity test has been added which, with the two tests for contact with blood, makes eleven tests.

a coacervate), an elastomeric matrix is formed. A commonly used cross-linking dose is 20 Mrad and the resulting matrix is designated X^{20}-poly(VPGVG). Poly(VPGVG), its cross-linked matrix and a number of related constructs are under consideration and development for a number of potential medical applications, i.e., (1) prevention of post surgical and post trauma adhesions, (2) drug delivery systems, (3) tissue reconstruction (e.g., synthetic ligaments and arteries), (4) wound coverings and dressings, (5) multiple applications for the eye, and (6) vascular stents.

While interesting modifications of the basic elastomeric polymer and matrix have been achieved such as introducing new structural properties, e.g., chemomechanical transduction [11,12], such as broadening ranges of elastic moduli from 10^5 to 10^8 dynes/cm^2 [13], and such as introducing cell attachment sequences [14,15] and useful enzyme sites, as yet the full range of biocompatibility results have to be reported for the parent elastomeric polypeptide poly(VPGVG) and its cross-linked matrix X^{20}-poly(VPGVG). The eleven tests for which the results are reported in summary form are (1) Ames mutagenicity test, (2) cytoxicity, (3) systemic toxicity, (4) intracutaneous toxicity, (5) muscle implantation, (6) acute intraperitoneal toxicity, (7) systemic antigenicity (British Pharmacopeia antigenicity test), (8) dermal sensitization (a maximization method of Magnusson and Kligman), (9) pyrogenicity, (10) Lee White clotting study, and (11) hemolysis. The complete raw data file and a copy of the final reports are in the archive files at North American Science Associates, Inc. (NAmSA®), 2261 Tracy Road, Northwood, Ohio 43619.

MATERIALS AND METHODS

Syntheses

Poly(GVGVP) was synthesized by two different methods for polymerization of the pentamer (GVGVP). Method A used nitrophenol, and Method B used EDCl and HOBt. In both cases BOC-Gly-Val-Gly-Val-Pro-OBzl was prepared as previously described [16].

Poly(Gly-Val-Gly-Val-Pro) (I): Method A (using nitrophenol). BOC-Gly-Val-Gly-Val-Pro-OH (36.92 *g*, 0.070 mol) was reacted with bis-PNPC (1.5 equiv.) in pyridine (290 mL). When the reaction was complete, the product was worked up by acid and base extractions to obtain 38.58 g (yield 85.00%) of BOC-Gly-Val-Gly-Val-Pro-ONp. This product (26.0 g, 0.040 mol) was deblocked using TFA and a one-molar solution of the TFA salt in DMSO was polymerized for 14 days using 1.6 equiv. of NMM as base.

The polymer was dissolved in pyrogen-free water, dialyzed using 3500 mol wt cut-off dialysis tubing and lyophilized. The product was base treated with 1N NaOH (2 equiv. per pentamer), dialyzed using 50 kD mol wt cut-off tubing for one week and lyophilized to obtain 9.49 (yield 57.83%) of I. This is designated Lot no. 1035b.

Poly(Gly-Val-Gly-Val-Pro) (II): Method B (using EDCI). BOC-Gly-Val-Gly-Val-Pro-OBzl (49.98 g, 0.081 mol) was hydrogenated and worked up by acid and base extractions to obtain 39.02 g (yield 91.41%) of BOC-Gly-Val-Gly-Val-Pro-OH. This product (2.11 g, 0.004 mol) was deblocked with TFA and a one-molar solution of the TFA salt in DMSO was polymerized for 8 days using EDCI (2 equiv.) as the polymerizing agent with HOBt (1 equiv.) and 1.6 equiv. of NMM as base. The polymer was dissolved in water, dialyzed using 3500 mol wt cut-off dialysis tubing and lyophilized. It was then dialyzed using 50 kD mol wt cut-off dialysis tubing for one week and lyophilized to obtain 1.09 g (yield 66.84%) of II. This is designated as Lot no. 1052.

When the Pro residue is placed at the carboxy terminus and nitrophenol is used in the polymerization, it has been shown to result in increased molecular weights [17]. The nitrophenol approach is the standard method that is used to polymerize the peptides. The EDCI method was used to avoid the nitrophenol group that could possibly remain in trace amounts. Materials made from these two methods were each run in the Ames mutagenicity test. Since both materials made, one by the nitrophenol and the other by the EDCI method, showed complete absence of mutagenicity, the nitrophenol method was chosen for producing the remaining poly(GVGVP).

The intermediate peptides were purified at each synthesis step. An important procedure, used in the preparation of the pentamer was the synthesis of the dipeptide BOC-Val-Pro-OBzl. This was dissolved in a minimal amount of ether, filtered, and then petroleum ether was added until the solution became cloudy. This cloudy solution was set aside for one day while crystal growth ensued. The crystals were then filtered to obtain pure BOC-Val-Pro-OBzl. BOC-Gly-Val-Gly-OBzl was treated in the same manner except that ethyl acetate was used instead of ether.

The high polymer poly(GVGVP) is soluble in water below 25°C. On raising the temperature aggregation occurs with settling to form the coacervate phase. The aggregation is monitored by the onset of turbidity. The temperature for onset of turbidity provides a critical assay as to the quality of the synthesis. The correct temperature for the onset of turbidity for Poly(Gly-Val-Gly-Val-Pro) is 25.5°C ± 1°C. ^{13}C NMR spectra to verify the synthesis are also routinely obtained. The presence of all requisite peaks and the absence of extraneous peaks is required to

verify the synthesis. This is done not only with the final product but also with the pentamer building blocks.

Preparation of the Cross-Linked Matrix

Poly(Gly-Val-Gly-Val-Pro) is dissolved in pyrogen-free water in the concentration of 250 mg/ml. The solution is then placed in a mold and centrifuged with the temperature maintained at 10°C below the transition. The temperature is then raised to 10°C above the transition over a period of one hour and then centrifuged for four more hours.

The coacervate phase is then checked for uniformity. If it does not have any irregularities such as bubbles, it is then γ-irradiated with a 20 Mrad dose to form the cross-links which result in an insoluble matrix. The molds are then opened in a laminar flow hood using sterile conditions and placed in a tube containing sterile water. The tube is then sealed.

In the test procedures, conducted by NAmSA®, that required an extract to be obtained of the test material, the material was extracted at 5°–15°C for 24 hours using sterile 0.9% sodium chloride USP solution. The reason for this adjustment in the protocol was that the material is in a swollen state (90% water) at this temperature, which allows the extraction of the material to be most effective. At 37°C the material contracts with extrusion of water and is not as readily extractable. Accordingly physiological temperature would be a less effective condition for extraction. This protocol ensured that everything was present in the extract that could cause a reaction in the tests used.

RESULTS

Ames Mutagenicity Test

1. This mutagenicity test, developed by Ames et al. [18,19], determines the rate of reversion to the wild type state of non-histidine dependence of five specifically constructed histidine-dependent mutant strains of *Salmonella typhimurium*, namely TA98, TA100, TA1535, TA1537 and TA1538. It is a widely used test for the determination of the mutagenic and thus potential carcinogenic propensity of the test article. A quantity of 0.1 gram of the poly(GVGVP) in 10 ml of DMSO was the test solution and DMSO alone was the negative control. Since the concern for possible mutagenicity is not only due to poly(GVGVP) but due to traces of chemicals, particularly the reactive aromatic chemicals that are used in the coupling reactions, two different syntheses

were tested, one in which *p*-nitrophenol was used in the polymerization and a second in which a carbodiimide and hydroxybenzotriazole were used. These are listed as Lot nos. 1035b and 1052, respectively. These poly(GVGVP) syntheses were initially found to be non-inhibitory to the growth of the tester strains of *Salmonella typhimurium*. As will be seen by examining Tables 1 and 2, the test sample is as innocuous as the negative DMSO solvent control whereas the well-known and often used Dexon® causes a dramatic increase in numbers of revertant colonies. This favorable outcome for the mutagenicity test suggests that carcinogenicity studies will not be necessary.

Cytotoxicity–Agarose Overlay

2. Using the agarose overlay approach a 1 sq. cm piece of X^{20}-poly(GVGVP) is placed over a monolayer of L-929 mouse fibroblast cells. Following incubation for 24 h at 37°C, there was no evidence of cell death below the article and there was no zone of lysis adjacent to the 1 cm^2 piece of X^{20}-poly(VPGVG). Quoting from the NAmSA® report, the "test article was *non-toxic* for L-929 mouse fibroblast cells under the above described test parameters."

Acute Systemic Toxicity

3. Twenty-four hour extracts of a 55.7 cm^2 sheet of X^{20}-poly(GVGVP) at temperatures between 5 and 15°C using 19 mL of 0.9% NaCl were injected intravenously into mice and compared to control solutions (extracts without test article). Two groups of five mice were used, one group for the extract and one for the control, and the mice were observed immediately after injection, at 4 h, 24 h, 48 h and 72 h. Quoting from the NAmSA® report, "There were no signs of toxicity."

Intracutaneous Toxicity

4. A 55.7 cm^2 sheet of X^{20}-poly(GVGVP) was extracted in a plastic vial for 24 h at 5 to 15°C with 19 mL of 0.9% NaCl. The extract was warmed to room temperature and 0.2 mL quantities were injected intracutaneously into five separate clipped sites on the right side of the back of each of two New Zealand White rabbits and control solutions were injected into five separate sites on the left side of each of the rabbits. The injection sites were examined for erythema (redness) and edema (swelling) at 24, 48 and 72 h after injection. As may be seen in Table 3 neither erythema nor edema was detected.

Table 1. Ames mutagenicity test. Plate incorporation assay of poly(GVGVP), Lot no. 1035b.

Salmonella typhimurium Tester Strains:	TA98	TA100	TA1535	TA1537	TA1538
	Number of Revertant Colonies (Average of Duplicate Plates)				
DMSO (– control)	20	78	9	6	7
DMSO test article solution	16	64	9	4	7
DMSO w/S-9 (– control)	17	90	8	8	10
DMSO test article solution w/S-9	15	76	12	6	6
Dexon* 1 mg/ml (+ control)	2144	1904	N/A	1656	N/A
Dexon* 1 mg/ml w/S-9 (+ control)	2000	1908	N/A	1680	N/A
Sodium azide 0.1 mg/ml (+ control)	N/A	N/A	2016	N/A	N/A
Sodium azide 0.1 mg/ml w/S-9 (+ control)	N/A	N/A	1832	N/A	N/A
2-nitrofluorene 1 mg/ml (+ control)	N/A	N/A	N/A	N/A	3720
2-nitrofluorene w/S-9 (+ control)	N/A	N/A	N/A	N/A	3792
2-aminofluorene 0.1 mg/ml (+ control)	N/A	96	N/A	N/A	34
2-aminofluorene w/S-9 (+ control)	N/A	3203	N/A	N/A	4793

N/A = Not applicable

Note: In no case was there a two-fold or greater increase in the reversion rate of the tester strains in the presence of the test article solution.

Table 2. Ames mutagenicity test. Plate incorporation assay of poly(GVGVP), Lot no. 1052.

Salmonella typhimurium Tester Strains:	TA98	TA100	TA1535	TA1537	TA1538
	Number of Revertant Colonies (Average of Duplicate Plates)				
DMSO (– control)	20	78	9	6	7
DMSO test article solution	19	73	10	5	6
DMSO w/S-9 (– control)	17	90	8	8	10
DMSO test article solution w/S-9	18	84	9	9	9
Dexon[5] 1 mg/ml (+ control)	2144	1904	N/A	1656	N/A
Dexon[6] 1 mg/ml w/S-9 (+ control)	2000	1904	N/A	1680	N/A
Sodium azide 0.1 mg/ml (+ control)	N/A	N/A	2016	N/A	N/A
Sodium azide 0.1 mg/ml w/S-9 (+ control)	N/A	N/A	1832	N/A	N/A
2-nitrofluorene 1 mg/ml (+ control)	N/A	N/A	N/A	N/A	3720
2-nitrofluorene w/S-9 (+ control)	N/A	N/A	N/A	N/A	3792
2-aminofluorene 0.1 mg/ml (+ control)	N/A	94	N/A	N/A	34
2-aminofluorene w/S-9 (+ control)	N/A	3208	N/A	N/A	4793

N/A = Not applicable

Note In no case was there a two-fold or greater increase in the reversion rate of the tester strains in the presence of the test article solution

Table 3. Intracutaneous toxicity of saline extracts of X^{20}-poly(GVGVP).

Extract	Rabbit No.		24 HR ER	24 HR ED	48 HR ER	48 HR ED	72 HR ER	72 HR ED
Sodium Chloride (SC)	56711	Test	0	0	0	0	0	0
		Control	0	0	0	0	0	0
	57174	Test	0	0	0	0	0	0
		Control	0	0	0	0	0	0

KEY

ER = Erythema
0 = None
1 = Barely perceptible
2 = Well defined
3 = Moderate
4 = Severe

ED = Edema
0 = None
1 = Barely perceptible
2 = Well defined
3 = Raised 1 mm
4 = Raised >1 mm

Rating (Test – Control)
0–0.5 Acceptable
0.6–1.0 Slight
>1.0 Significant

Muscle Implantation

5. Two adult New Zealand White rabbits were each implanted at four sites in the right paravertebral muscle by needle injection of fragments arising from a 0.7 mm × 1 mm × 10 mm piece of X^{20}-poly(GVGVP). The material was assumed to remain sterile from the γ-irradiation. Controls of USP control plastic were injected as 1 mm × 10 mm strips in two sites in the left paravertebral muscle of each rabbit. After 7 days the animals were sacrificed, and the test and control implantation sites were compared macroscopically and microscopically. Macroscopically, "The reaction was *not significant* as compared to the negative control" (see Table 4a). With ratings of non-irritant, slight irritant, moderate irritant and severe irritant, microscopically, "The reaction was *a slight irritant* as compared to the negative control implant material" (see Table 4b). Of all the tests, as this is the only test where a significant albeit slight irritant effect has been observed, it will be considered in more detail in the Discussion section.

Acute Intraperitoneal Toxicity Study

6. The animal model to evaluate relative acute toxicity, historically, is the rat. Ten 1 cm × 1 cm pieces of X^{20}-poly(GVGVP) were implanted

Table 4a. Muscle implantation test of X^{20}-poly(GVGVP) (macroscopic examination).

Rabbit No.	Article	Test	Control
	1	0	0
56512	2	0	0
2.7 kg	3	0	
	4	0	
	1	0	0
56639	2	0	0
2.5 kg	3	0	
	4	0	
Average		0.0	0.0

Scoring Key		Reaction Index	
Score	Capsule Formation	Average (test) – Average (control) = 0.0	
0	None noted	0-0.5	Not significant
1	Up to 0.5 mm	0.6-1.0	Trace
2	0.6 to 1.0 mm	1.1-2.0	Slight
3	1.1 to 2.0 mm	2.1-3.0	Moderate
4	>2.0 mm	>3.0	Marked

Table 4b. Muscle implantation test of X^{20}-poly(GVGVP) (microscopic examination).

	Test		Control	
Animal No.:	56512	56639	56512	56639
Observation (0-4)				
Inflammation				
Polymorphonuclear	0	0	0	0
Lymphocytes	1	1	1	1
Plasma cells	0	0	0	0
Macrophages	1	1	1	1
Giant cells	2	3	1	0
Necrosis	0	0	0	0
Subtotal (×2)	18		10	
Fibroplasia	2	2	0	0
Fibrosis	2	2	2	2
Fatty infiltrate	0	0	0	0
Subtotal	8		4	
Difference (Test # – Control)				
Total Test 26 – Control 14 = 12 (slight irritant)				
Foreign debris	3	4	0	0
Number sites examined	4	4	2	2

Score Guide
(Test – Control)
0 = Non-irritant
1-15 = Slight
16-30 = Moderate
>30 = Severe
for a two rabbit test

surgically into the peritoneal cavity of each of ten rats and left in place for 28 days. The animals were euthanatized and examined for signs of systemic toxicity and for presence of remaining test material. The general health of all of the rats was good with no evidence of adverse behavior or illness. Upon gross autopsy at termination all rats appeared macroscopically normal. The test articles were found in nine rats but not in one. In the preimplant condition, the test article is soft, compliant and optically clear and the appearance of all recovered test articles was "very similar to pre-implant condition." Quoting from the conclusions of the NAmSA® report, "Under the conditions of this study, there was no evidence of acute systemic toxicity in rats implanted intraperitoneally for four weeks with X^{20}-poly(GVGVP)."

Systemic Antigenicity Study

7. Solutions of poly(GVGVP), Lot no. MR183, were injected intraperitoneally into six guinea pigs three times a week, every other day. Following the initial injection at fourteen days, three guinea pigs were challenged by an IV injection and the other three were IV injected at 21 days. This is the method described in *The British Pharmacopeia* to evaluate the antigenic and anaphylactic potential of the test article. Quoting from the NAmSA® report, "Anaphylactic signs would include face pawing, eye blinking, hyperactivity, lethargy, convulsions and death." "Under the conditions of this test, the test article would not be considered antigenic in the guinea pig; no significant reactions to the challenge were observed."

Dermal Sensitization Study (A Maximization Method) [20]

8. The animal model is the Hartley guinea pig strain with established susceptibility. With a ratio of 60 cm^2 to 20 mL of 0.9% sodium chloride USP solution (SC), X^{20}-poly(GVGVP) was extracted for 24 h at 5–15 °C to obtain the test solution extract. Fifteen albino guinea pigs, 10 test and 5 control, were clipped of hair each time for each injection and treatment site. In the ten test animals, Induction I involved three rows of intradermal injections (two per row) of (1) 0.1 mL of Freund's Complete Adjuvant (FCA), (2) 0.1 ml of SC test article extract, and (3) 0.1 mL of a 1:1 suspension of the SC test article extract and FCA. The 5 control animals were retained for use in the challenge phase. Induction II occurred one week after the injections with ~1 gram of 10%

sodium lauryl sulfate suspension in petrolatum massaged into the skin in the area of the injections to provoke a mild acute inflammation and gently wiped off 24 h later. Then 0.3 mL of the SC test article extract saturating a 2 × 4 cm filter paper was applied to the injection sites and retained there for 48 h.

The primary challenge, which occurred 13 days after the Induction II patch application, involved 0.3 mL of the SC test article extract or SC control saturating a non-woven cotton disc applied on the left flank and 2 cm × 2 cm patches of X^{20}-poly(GVGVP) applied to the right flank using the 10 test animals and the 5 control animals. The patches were maintained in position for 24 hours and observations were made at 24, 48, 72 and 96 h after primary challenge patch removal. The results of the primary challenge are given in Table 5. Details of the scoring are given in Appendix A. The rechallenge, allowing for a more conclusive evaluation of the sensitizing potential, occurred 7 days after the initial challenge by using a complete new set of patches and different topical sites. The results of the rechallenge are given in Table 6. Quoting the conclusion of the NAmSA[8] report, "Under the conditions of this study, neither the test article nor the SC test article extract showed significant evidence of causing dermal sensitization in the guinea pig."

Rabbit Pyrogen Study

9. Ten pieces of X^{20}-poly(GVGVP), 1 cm^2, were covered with 400 mL of sterile, nonpyrogenic saline. A single dose of 10 ml/kg was intravenously injected into a rabbit ear vein of each of three rabbits and the temperatures were electronically monitored rectally. A five-rabbit retest was run for which the mean maximum rise was 0.1°C. Quoting the conclusion of the NAmSA[9] report, "Under the conditions of this study, the test article solution was not considered pyrogenic."

Lee White Clotting Study

10. The clotting time of one mL fresh canine blood in each of six siliconized tubes was determined in the presence (3 tubes) and absence (3 tubes) of 0.5 cm × 0.5 cm squares of X^{20}-poly(GVGVP). After one minute the tubes were gently tilted at 30 second intervals. Clotting time was when withdrawal of a needle showed a firm clot. This was repeated three times for a total of 18 tubes, 9 test and 9 control. Quoting the conclusion of the NAmSA[8] report, "Under the conditions of this

Table 5. Guinea pig sensitization, dermal reactions—primary challenge, for X[20]-poly(GVGVP).

Animal Number	OBS	Hours Following Patch Removal: 24 Site A	24 Site B	24 Site C	48 Site A	48 Site B	48 Site C	72 Site A	72 Site B	72 Site C	96 Site A	96 Site B	96 Site C
1 Test	ER	0	1	0	0	0	0	0	0	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
2 Test	ER	0	0	1	0	0	0	0	0	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
3 Test	ER	0	1	1	0	1	0	0	1	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
4 Test	ER	1	0	1	1[a]	1	0	1[a]	1	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
5 Test	ER	0	0	1	1[a]	0	0	1[a]	0	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
6 Test	ER	1	0	1	0	0	0	0	0	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
7 Test	ER	1[a]	0	1	0	0	0	0	0	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
8 Test	ER	1[a]	0	1	1[a]	1	0	0	1	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
9 Test	ER	0	0	1	0	1	1	0	1	1	0	1	1
	ED	0	0	0	0	0	0	0	0	0	0	0	0
10 Test	ER	1[a]	1	1	0	1	1	0	1	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
11 Control	ER	0	0	1	0	0	0	0	0	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
12 Control	ER	1	1	1	0	0	0	0	0	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
13 Control	ER	1	1	1	0	0	1	0	0	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
14 Control	ER	0	0	1	0	0	1	0	0	1	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
15 Control	ER	0	0	1	1[a]	0	0	1[a]	0	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0

[a]Apparent mechanical trauma that extended beyond the site

ER = erythema, ED = edema (Evaluation as in key of Table 3.)

Site A = Upper left flank = SC control vehicle
Site B = Lower left flank = SC test article extract
Site C = Right flank = 2 × 2 cm patch of test article

Table 6. Guinea pig sensitization, dermal reactions—rechallenge, for X[20]-poly(GVGVP).

Animal Number	OBS	Hours Following Patch Removal											
		24			48			72			96		
		Site A	Site B	Site C	Site A	Site B	Site C	Site A	Site B	Site C	Site A	Site B	Site C
1 Test	ER	0	1[b]	1[b]	0	1	0	0	1	0	0	1	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
2 Test	ER	0	0[c]	0	0	1	0	0	1	0	0	1	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
3 Test	ER	0	1	1	0	1	1[b]	0	1	0	0	1[c]	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
4 Test	ER	0	1	1[b]	0	1	0	0	1	0	0	1	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
5 Test	ER	0	1	0	0	1	0	0	1	0	0	1	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
6 Test	ER	0	0	0	0	0	0	0	0	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
7 Test	ER	0	0	1	0	0	0	0	0	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
8 Test	ER	0	0	1	0	0	0	0	0	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
9 Test	ER	0	0	1[b]	0	0	0	0	0	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
10 Test	ER	0	0	1	0	1	1	0	1	1[b]	0	1	1
	ED	0	0	0	0	0	0	0	0	0	0	0	0
11 Control	ER	0	0	1	0	0	1	1	0	0	1	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
12 Control	ER	0	0	0	0	0	0	0	0	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
13 Control	ER	0	1[c]	1[c]	0	1	1[b]	0	1	0	0	1	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
14 Control	ER	0	0	0	0	0	0	0	0	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0
15 Control	ER	0	0	0	0	0	0	0	0	0	0	0	0
	ED	0	0	0	0	0	0	0	0	0	0	0	0

[a]Apparent mechanical trauma that extended beyond the site
[b]Apparent mechanical trauma around test site
[c]Scratch on test site
[d]Pinpoint area(s) of redness
ER = erythema, ED = edema (Evaluation as in key of Table 3 or Appendix A.)
Site A = Dorsum (cephalic) = SC control vehicle
Site B = Dorsum (caudal) = SC test article extract
Site C = Right flank = 2 × 2 cm patch of test article

test, there were no significant differences between clotting times of test and control tubes. All clotting times were within normal ranges for the dog" *(3rd Edition, Veterinary Hematology,* Schalm, Jain, and Carroll).

Hemolysis Test *(in vitro)*

11. A volume of 37 mL of 0.9% sodium chloride solution at 5–15°C was used to extract a 112 cm^2 patch of X^{20}-poly(GVGVP) for 24 h. A quantity of 10 mL of extract as test and 10 mL of 0.9% sodium chloride as control was placed in each of two tubes, for a total of four tubes. To each of these was added 0.2 mL of rabbit blood that had been collected in a vacuum tube containing EDTA. The tubes were gently mixed by inverting, placed in a 37°C bath for 1 h and centrifuged for 10 minutes at 1000 × g or greater. The absorbance of this clear solution was determined at 545 nm. The results were 0% hemolysis. Quoting from the NAmSA® results, "After 1 h, there was no evidence of red blood cell lysis as determined spectrophotometrically."

DISCUSSION

In all eleven tests poly(GVGVP) and X^{20}-poly(GVGVP) fared as well as the negative controls with the exception of the muscle implantation in which a slight irritation was observed. For this test there has been a more extensive study completed [21] in which X^{20}-poly(GVGVP) sterilized by ethylene oxide gas was compared to a sham incision and to Dacron® and Dexon® implants for rabbit muscle implants examined at 3, 6, 12 and 24 weeks. Quoting from the abstract, "A comparison of the three different biomaterials and the sham at each time period demonstrated the most significant tissue reaction for the Dacron® while the least was for the polypentapeptide, X^{10}-poly(VPGVG), and the sham which showed normal healing." Significantly at 3 weeks there was no fibrous capsule adjacent to the X^{20}-poly(VPGVG) matrix. In the muscle implantation study summarized here there was a small fibrosis and fibroplasia reported (see Table 4b). In the implant studies when the test article is observed, it is seen as a uniform translucent material. This raises the question as to the origins of the debris noted in the present report and to the possibility of contamination with debris during the preparation of the material or the handling prior to implantation. Thus even this slight irritation may be an overstatement of the reaction to X^{20}-poly(GVGVP). This study is being repeated by NAmSA® with new material. In addition there are several earlier collaborative studies

*Table 7. Summary of biological test results for poly(VPGVG) and certain analogues.**

Test		Description	Test System	Results
1	Ames mutagenicity	Determine reversion rate to wild type of histidine-dependent mutants	*Salmonella typhimurium*	Non-mutagenic
2	Cytotoxicity	Agarose overlay, determine cell death and zone of lysis	L-929 mouse fibroblast	Non-toxic
3	Systemic toxicity	Evaluate acute systemic toxicity from an IV or IP injection	Mice	Non-toxic
4	Intracutaneous toxicity	Evaluate local dermal irritant or toxic effects by injection	Rabbit	Non-toxic
5	Muscle implantation	Effect on living muscle tissue	Rabbit	Favorable
6	IP implantation	Evaluate potential systemic toxicity	Rat	Favorable
7	Systemic antigenicity (BPAT)	Evaluate general toxicology	Guinea pigs	Non-antigenic
8	Sensitization (Kligman test)	Dermal sensitization potential	Guinea pigs	Non-sensitizing
9	Pyrogenicity	Determine febrile reaction	Rabbit	Non-pyrogenic
10	Clotting study	Whole blood clotting times	Dog	Normal clotting time
11	Hemolysis	Level of hemolysis in the blood	Rabbit blood	Non-hemolytic

*Reports from North American Science Associates (NAmSA)

that indicate biocompatibility in human cell cultures and in rat and rabbit soft and hard tissue sites [22–25].

CONCLUSIONS

The remarkable biocompatibility of poly(GVGVP) and its 20 Mrad γ-irradiation cross-linked matrix, X^{20}-poly(GVGVP), has been demonstrated by the series of independent test results summarized here. (See Table 7 for a summary of the biological test results.) As a soft, compliant, water-containing, biocompatible matrix that is innocuous to tissues, this bioelastic material is positioned to be considered directly in a number of medical applications, and with many different modifications possible selective new properties can be designed into the matrix. For example, cells do not adhere to the X^{20}-poly(GVGVP) matrix but on addition of specific cell attachment sequences to the polymerizing flask at useful ratios to the GVGVP pentamer, a matrix for selective cell attachment and adhesion has been developed [14,15]. Furthermore, since the matrix can be designed to undergo chemically induced swelling and contraction, the capacity of these bioelastic materials to carry out useful biological and medical functions can be anticipated.

NOMENCLATURE

BOC	tert-butyloxycarbonyl
OBzl	benzylester
bis-PNPC	bis(4-nitrophenyl)carbonate
EDCI	1-ethyl-3-dimethylaminopropylcarbodiimide
NaOH	sodium hydroxide
HOBt	1-hydroxybenzotriazole
DMSO	dimethylsulfoxide
NMM	*N*-methylmorpholine
TFA	trifluoroacetic acid

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APPENDIX A–DRAIZE† EVALUATION OF TOPICAL REACTIONS

Adapted from NAmSA® Report

	SCORE
Erythema and Eschar Formation (most predominant condition)	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4

Note: Test sites assigned to a "4" score for erythema require further description as to the extent of tissue injury.

Edema Formation (most predominant condition)	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well-defined by definite raising)	2
Moderate edema (raised approximately 1 millimeter)	3
Severe edema (raised more than 1 mm and extending beyond the area of exposure)	4

APPENDIX B–DERMAL SENSITIZATION ALLERGENICITY RATING

% Reacting	Classification
0%	Not a sensitizer
1–10%	Weak sensitizer
11–30%	Mild sensitizer
31–60%	Moderate sensitizer
61–80%	Strong sensitizer
81–100%	Extreme sensitizer

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Temperature of Polypeptide Inverse Temperature Transition Depends on Mean Residue Hydrophobicity

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For the many hydrophobicity scales for amino acid residues in protein and polypeptides,[1-11] there is reasonable agreement for a sequence in which Phe (F) > Ile (I) ≃ Leu (L) > Val (V) > Ala (A) > Gly (G). When these residues are substituted at position 4 within $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$, abbreviated poly(VPGVG), to give a structure written poly[f_X(VPGXG),f_V-(VPGVG)] where f_V is the mole fraction of pentamers with valyl residues at position 4 and f_X is the mole fraction of pentamers with a guest residue, X, at position 4 with $f_X + f_V = 1$, the temperature of a reversible aggregational transition in water (actually a phase transition described as a coacervation) is here demonstrated to be inversely dependent on the mean hydrophobicity, and in addition, the heat of the transition is found to be directly proportional to the mean residue hydrophobicity in studies where the temperature and heat of the transition were determined by differential scanning calorimetry (DSC). With the use of transition temperature to determine relative hydrophobicity having been established in this molecular system, data for Trp (W), Tyr (Y), and Met (M) complete the values for residues comprising the apolar half of the natural amino acids in this first hydrophobicity scale based on a physical property so integral to the process of protein folding and assembly. Importantly, the transition temperature, in this relatively simple polypeptide (pro-

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(1) Prior to the present report, relative hydrophobicities were derived from relevant but indirect approaches. For example, the Nozaki and Tanford scale was based on relative solubilities of amino acids in organic solvents;[2] the Bull and Breese scale utilized surface tension of amino acid solutions;[3] Hopp and Woods used the correlation of hydrophilicity with antigenic determinants;[4] a number of scales derive from the distribution of residues buried within or on the surface of globular proteins;[5-10] and more recently and more closely related to the present report, the physically relevant partial molar heat capacities of peptide moieties and amino acid side chain equivalents have been used but with limitations due to low solubility of the side chains of the Ala, Val, Leu, and Ile amino acids and due to the assumed additivity of polar and apolar components.[11]

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tein-based polymer) system, which is nonetheless capable, when suitably substituted and cross-linked, of efficient thermomechanical and chemomechanical free energy transductions,[12] can now be used to study the effect of various perturbations on the expression by hydrophobicity, such as polar residues, salts in solution, isotope effects (e.g., ^2H_2O), organic solvents, etc.

Repeat peptides of elastin such as $(VPGVG)_n$ and the related $(VAPGVG)_n$ exhibit an increase in order both intramolecularly and intermolecularly when the temperature is raised through a critical temperature range, that is, they exhibit inverse temperature transitions.[13] This is most unambiguously demonstrated by cyclo$(VAPGVG)_2$, which reversibly crystallizes when the temperature is raised and dissolves when the temperature is lowered through a transition range.[14] Another informative cyclic analogue which aggregates when the temperature is raised is cyclo$(VPGVG)_3$. The crystal structure of this analogue is notable (1) due to a substantial water content, with the water not occurring between molecules but rather within the cylindrical stack of cyclic molecules; (2) due to the extensive hydrophobic interactions between molecules, within and between stacks;[15] and (3) due to the conformation of the cyclic molecule being a cyclic conformational correlate[16] of linear poly(VPGVG), which itself self-assembles, when the temperature is raised, into fibers comprising fibrils which are bundles of parallel aligned twisted filaments formed from the supercoiling of loose hydrophobically assembled helical structures of poly(VPGVG) called β-spirals.[13,17] A wide range of physical methods have been used to characterize the increase in order intra- and intermolecularly as the temperature is raised through the folding and aggregational transition.[18] Now that an increase in polypentapeptide order increasing temperature is established, it is natural to look to ordered water molecules becoming disordered to achieve the net entropy increase when the temperature is raised through the transition in keeping with the second law of thermodynamics. Furthermore, since the secondary structure of poly(VPGVG) does not change on passing through the transition,[19] it becomes obligatory that waters of hydrophobic hydration be considered.

One direct check of this rationale is to synthesize a more hydrophobic analogue in which the conformation is the same as that of poly(VPGVG) before the transition and changes to the same folded conformation as that of poly(VPGVG) after the transition. This has been achieved with the poly(IPGVG) which has an added CH_2 moiety per pentamer but which retains important β-branching at position 1.[20,21] The result is that the increase in hydrophobicity lowers the temperature of the transition and increases the heat of the transition.[22] The interpretation is that the increase in endothermic heat required to drive the transition reflects the energy required to destructure the greater numbers of waters of hydrophobic hydration.

Because β-branching provides an important hydrophobic interaction during folding, i.e., a $Val_i^1\gamma CH_3...Pro_i^2\delta CH_2$ interaction identified by the nuclear Overhauser effect,[21,23] this position does not lend itself to many isomorphic substitutions, but position 4 does. [For example, poly(APGVG), rather than reversibly forming a viscoelastic coacervate, irreversibly forms a granular precipitate when the temperature is raised.][24] The general formula poly-$[f_X(VPGXG),f_V(VPGVG)]$ provides a series of polypentapeptides of varying hydrophobicity in which the temperature of the transition and the heat of the transition could provide a hydrophobicity scale, a scale derived directly from hydrophobically driven folding and assembly of interest to protein structure and function.

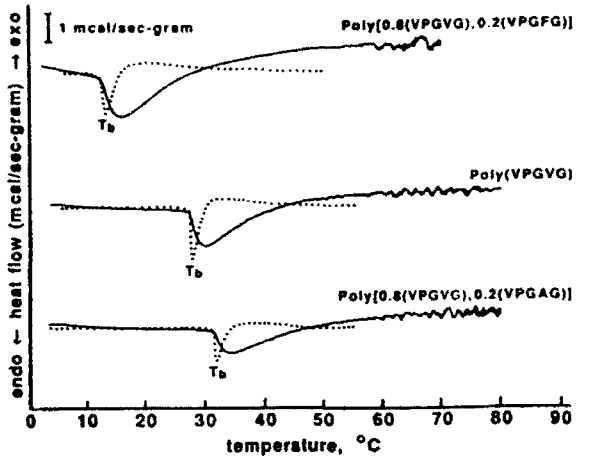


Figure 1. DSC thermograms (solid line) and their derivatives with respect to temperature (dotted line) of three polypentapeptides in H_2O. The scan rate was 0.23 °C/min. The peptide concentration is 40 mg/mL.

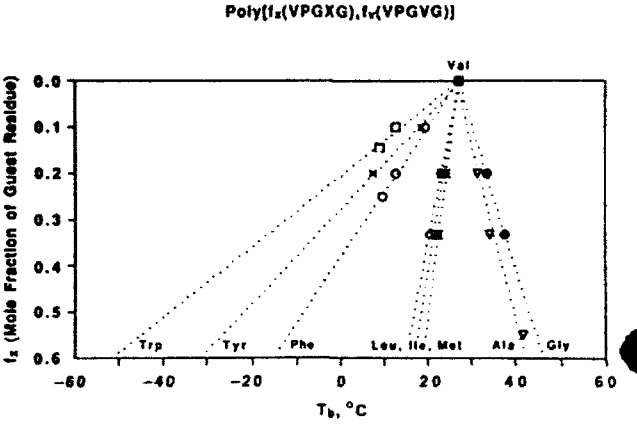


Figure 2. The dependence on mole fraction of guest residue, f_X, of the temperature (T_b) of an inverse temperature transition, given as the extremum on the derivative with respect to temperature of the DSC scan for 19 different polypentapeptides with the general composition, poly-$[f_X(VPGXG),f_V(VPGVG)]$. The dependence of f_X is seen to be very nearly linear such that the values may be extrapolated to a reference value for f_X to give a comparison of hydrophobicities.

Reported here are DSC data on 19 polypentapeptides of molecular weight greater than 50 000 Da. The synthesis and verification of these 19 polymers will be presented elsewhere; they constitute a fraction of more than 200 high-polymer syntheses that provide the necessary knowledge base for confidence in the results of Figures 1 and 2. Representative DSC data for three compositions are given in Figure 1, where the transition temperature is defined as T_b from the extremum of the derivative curve. The heats of the transition were determined as previously described.[22] Data for the 19 polypentapeptides are plotted in

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Figure 2 as the mole fraction of guest residue, f_X, versus T_b. Compositions with values of T_b below 0 °C cannot be studied because of inability to achieve the dissolved state below the freezing point of water.

From this data the relative hydrophobicities are seen to be Trp > Tyr > Phe > Leu ≃ Ile ≃ Met > Val > Ala > Gly. While the accuracy with which the experimental heat (ΔH) of the transition can be determined is less than that of the temperature, a similar scale is obtained from the difference in heats due to the substitution, i.e., $\delta\Delta H(X) = \Delta H(VPGXG) - \Delta H(VPGGG)$ with extrapolation to $f_X = 1$. The results of the $\delta\Delta H$ in kilocalories/mole are W (6.2) > F (5.6) > Y (4.6) > L (3.9) > I (3.6) > V (2.0) > M (1.8) > A (0.4) > G (0.0). Clearly, Trp comes out to be the most hydrophobic in this functional scale, and interestingly there is an interchange between the Tyr and Phe order when temperatures and heats of the transition are compared. It may ultimately be more appropriate to use an entropy scale, i.e., $\delta(\Delta H/T)$. From the practical side, when the temperature scale is used, it becomes possible to choose a combination of residues tha. gives any desired value for T_b from ~0 °C to about 60 °C. This sets the stage for the proper consideration of polar groups including the demonstration that COO^- is very much more polar than COOH[12,25,26] and allows that the temperature and heat of the transition may be used to determine the effects of other perturbations, such as NaCl and other salts, urea, guanidine hydrochloride, ethylene glycol and other alcohols, dimethyl sulfoxide, etc., on the expression of hydrophobicity.

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Synthetic Polypeptide Sleeve for Strabismus Surgery

Frederick J. Elsas, MD, D. Channe Gowda, PhD, and Dan W. Urry, PhD

ABSTRACT

A synthetic polypentapeptide sleeve was placed around the superior rectus muscle of five New Zealand white rabbits in hopes of preventing postoperative fibrous scarring. Two forms of the polypentapeptide were used. No significant inflammation or scarring occurred with either form of the polypentapeptide when compared to controls. One form elicited a fibrous membrane surrounding the sleeve within 2 weeks. The other elicited no such reaction after 2 months. The latter form of the polypentapeptide may be useful in preventing scarring following strabismus surgery.

INTRODUCTION

Contemporary methods for strabismus surgery attempt to minimize postoperative scarring around the extraocular muscles. These include preservation of the muscle capsule, control of bleeding, minimal use of cautery, and avoidance of orbital fat. Fibrovascular and fibrofatty scarring can occur with poor surgical technique or following hemorrhage or suture reaction. This scarring is particularly common in patients having multiple strabismus procedures or retinal detachment surgery. When present, the scarring between muscle, sclera, and conjunctiva can restrict eye movement severely; it may even cause misalignment of the eyes.

Strabismus surgery directed at removal of scar tissue with injection of periocular corticosteroids can improve eye position but cannot always prevent recurrence of scarring and adhesions. In the past, tubes or sleeves made of Supramid Extra®, silicone, and polyglactin 910[1-4] mesh have been used with varying success to try to prevent scarring and restriction to eye movement. These have largely been abandoned. Supramid Extra® and silicone can extrude or induce formation of a fibrous sleeve around themselves, thus limiting eye movement. Polyglactin mesh does not prevent ingrowth of small vessels into the muscle and may induce a reaction similar to a suture reaction.

Recently, a potentially degradable, synthetic polypentapeptide has been developed which mimics the amino acid sequence found in naturally occurring elastin. It appears to be minimally antigenic and shows promise in preventing the occurrence of scar tissue in biologic systems.[5,6] We report herein the implantation of two forms of this polypentapeptide around the superior rectus muscles of rabbits in an attempt to minimize postoperative scarring and adhesion formation.

MATERIALS AND METHODS

Five New Zealand white rabbits weighing approximately 3 kg were anesthetized with a mixture of ketamine, 30 mg/kg, and acepromazine, 0.75 mg/kg. The right eye of each animal was used as a control. A fibrous adhesion between the superior rectus and sclera was created using a modification of the Sondhi method.[4] The superior rectus was isolated through a limbal incision and the superior oblique disinserted. One 6-0 polyglactin 910 (Vicryl) suture was imbricated through the muscle's insertion, and the muscle was disinserted. A 3- × 3-millimeter lamellar sclerectomy was created with a #69 Beaver blade just posterior to the muscle's insertion, and the muscle capsule

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FIGURE 1: Eight weeks after implantation, a dense scar binds the superior rectus to the site of the lamellar sclerectomy in the control eye (arrow) (hematoxylin and eosin; original magnification × 100).

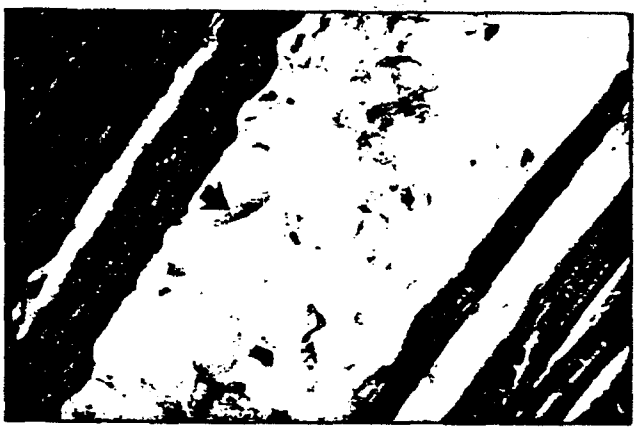


FIGURE 2: Eight weeks after implantation, at the site of the sclerectomy, the poly (VPGVG) sleeve (arrow) is interposed between the muscle (left) and sclera (right). The sleeve is artifactitiously disrupted (hematoxylin and eosin, original magnification × 400).

overlying the sclerectomy was removed. The muscle was then resutured to the original insertion, and the conjunctiva closed with four 6-0 plain gut sutures.

The left eye was the experimental eye in each animal. In three animals, a 12-millimeter long × 6-millimeter wide rectangle of gamma irradiation cross-linked poly (Valine-Proline-Glycine-Valine-Glycine), poly (VPGVG), was wrapped around the muscle and closed with three 6-0 polyglactin 910 (Vicryl) sutures to form a sleeve. In two animals, 6-millimeter long tubes of gamma irradiation cross-linked poly (3 [Valine-Proline-Glycine-Valine-Glycine], [Valine-Proline-Glycine-Phenylalanine-Glycine]), poly [3(VPGVG),(VPGFG)], were fashioned from tubes of the material, eliminating the need for the polyglactin 910 sutures to close the sleeve. In all experimental eyes, a lamellar sclerectomy identical to the that for the control eyes was created posterior to the muscle insertion and a section of muscle capsule was removed. The polypentapeptide material had been prepared under sterile conditions,[5] was soaked in gentamicin ophthalmic solution for 30 minutes, and rinsed in sterile saline prior to implantation.

The animals were examined daily for 1 week and thereafter weekly until they were killed. The three animals receiving the poly (VPGVG) sleeve were killed at 1, 6, and 8 weeks. The two animals receiving the poly [3 (VPGVG),(VPGFG)] sleeve were killed at 2 and 3 weeks. The animals were euthanized with barbiturates. The conjunctiva overlying the superior rectus in the control and experimental eyes was examined. The eyes were enucleated, taking care to preserve at least 8 mm of superior rectus and the sleeve material. All specimens were examined histopathologically.

RESULTS

The superior rectus muscle in the control eye of each animal was apposed tightly to the sclera at 1 week. Histopathologic examination showed a dense fibrovascular scar binding muscle to sclera in the 8-week specimen (Fig 1). The conjunctiva overlying the muscle did not adhere to it.

Both the poly (VPGVG) and the poly [3 (VPGVG), (VPGFG)] sleeves were well tolerated by the rabbit model. The conjunctiva of the experimental eyes remained white and quiet after the initial mild inflammation resulting from the procedure subsided in several days. The 8-week specimen containing the poly (VPGVG) sleeve extruded through the conjunctiva after the polyglactin 910 sutures holding the sleeve dissolved. Even then, the eye was not inflamed.

In all animals receiving the poly (VPGVG) sleeve, the conjunctiva could be separated freely from the surface of the superior rectus. No membrane encircled the sleeve material. Histopathologic examination of these specimens revealed no significant inflammation either on the conjunctival surface of the muscle or at the interface between muscle, sleeve, and lamellar sclerectomy (Fig 2). The poly (VPGVG) remained intact throughout the 8-week course of the experiment.

The eyes of the two animals receiving the poly [3 (VPGVG),(VPGFG)] tubular sleeve also remained uninflamed. The conjunctiva could be separated easily from the muscle. A glistening capsule formed around this sleeve within 2 weeks after its implantation (Fig 3). Histopathologic examination revealed this fibrous capsule to be confined to the sleeve material (Fig 4). No significant inflammation occurred at the surface of the muscle.

DISCUSSION

Two forms of a synthetic polypeptide analog of naturally occurring elastin were evaluated in a rabbit experimental model for their ability to diminish the scarring that can occur after strabismus surgery. In control eyes, dense scars formed between the muscle and sclera. Sleeves of poly (VPGVG) elicited an insignificant inflammatory reac-



FIGURE 3: A glistening fibrous membrane (arrow) surrounds the poly [3 (VPGVG),(VPGFG)] tube 2 weeks after implantation.

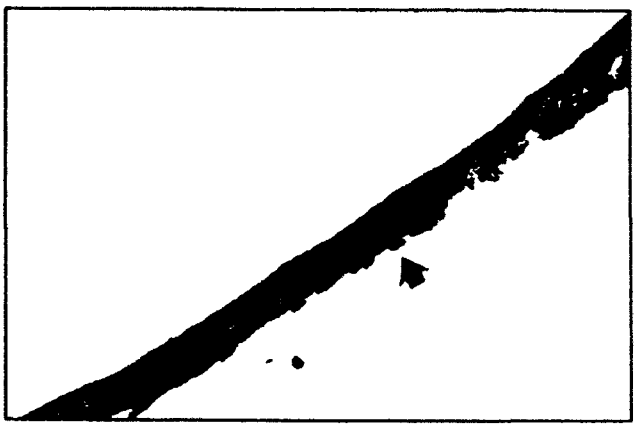


FIGURE 4: The fibrous membrane surrounding the poly 3 (VPGVG), (VPGFG) tube (arrow). The polypentapeptide is to the left but stains poorly (hematoxylin and eosin; original magnification × 400).

tion and were well tolerated by the animal. No fibrous membrane formed around the sleeve.

Poly [3(VPGVG), (VPGFG)] similarly caused little inflammation. However, a dense fibrous tissue membrane formed rapidly around the implanted material. Substituting phenylalanine for one valine residue is sufficient to cause this change in reactivity. This fibrous capsule could limit eye movement and makes this material unsuitable for use in strabismus surgery.

Further work is proceeding on developing a poly (VPGVG) sleeve. Tubular molds are now being evaluated which will permit fabrication of small tubes of the material. This will eliminate the need for closing the sleeve with sutures. Modifications are being made in the structure of the pentapeptide to permit it to dissolve in the subconjunctival space. This would eliminate the likelihood of extrusion months after surgery and would prevent the sleeve from limiting eye movements.

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MEDICAL APPLICATIONS OF BIOELASTIC MATERIALS

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ABSTRACT

Repeating peptide sequences that occur in mammalian elastic fibers exhibit interesting properties of entropic (ideal) elasticity and of folding and assembling on raising the temperature. When correctly understood, these properties make it possible to design and synthesize elastomeric polypeptides capable of exhibiting additional properties and functions with both medical and non-medical applications.

For medical applications, it is first necessary to demonstrate biocompatibility. This has been done for the parent elastic protein-based polymer, poly(Val^1-Pro^2-Gly^3-Val^4-Gly^5) or poly(VPGVG), which exhibits an extraordinary biocompatibility as the polymer and as the γ-irradiation cross-linked elastomeric matrix, designated as X^{20}-poly(VPGVG). As cells do not adhere to this matrix and as no fibrous capsule forms around it when implanted, the matrix and other states of the material have potential for use in the prevention of post-operative, post-trauma adhesions. Specific studies underway include a contaminated peritoneal model using the rat, a strabismus surgery model using the rabbit eye, and just beginning a total artificial heart model as a bridge to transplantation using the calf.

Because cell attachment sequences can be introduced into the matrix which result in cell adhesion, spreading and growth to confluence, such modified elastomeric matrices become candidates for use in a wide range of possible tissue reconstructions. In general, the concept is to design a functional scaffolding for a particular tissue into which the appropriate cells can migrate and within which they function and remodel the prosthetic material into a natural tissue with slow degradation and removal of the temporary synthetic scaffolding.

These compliant, biocompatible matrices also have significant potential for use in drug delivery. This is made especially attractive because, in addition to being able to introduce chemical clocks that would control their rate of degradation as desired, they

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can be designed to swell or to contract in response to a relevant chemical signal which may be associated with a particular diseased state.

This capacity to design the matrices to contract and relax in response to a chemical signal (i.e., to function in chemomechanical transduction) and even to carry out more diverse free energy transductions known to be important in living organisms suggests further dynamic roles in their potential medical applications.

INTRODUCTION

GENERAL BACKGROUND

Elastomeric Polypeptides that Increase Order on Increasing the Temperature: Bioelastic materials have their origins in repeating sequences that occur in the mammalian elastic fiber [1-3]. The most prominent repeating sequence is $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$ with n = 11 in bovine elastin [3]. High polymers of this sequence, poly(VPGVG), have been chemically and microbially synthesized [4-6]; they exhibit the interesting property of being soluble in water at low temperatures, below 25°C, but then aggregate into a more-ordered, viscoelastic state, called a coacervate, on raising the temperature to 37°C [7]. This process of increasing order on increasing the temperature is called an inverse temperature transition [8].

Matrices for the Conversion of Thermal Energy Into Motion: When the coacervate state is cross-linked, it forms elastomeric matrices with elastic moduli similar to those of the elastic fiber. When 20 Mrads of γ-irradiation are used to achieve cross-linking, the resulting elastic matrix is designated X^{20}-poly(VPGVG). A small sheet of elastic matrix is seen in Figure 1. The cross-linked matrix exhibits the inverse temperature transition behavior by contracting on raising the temperature to 37°C and swelling on lowering the temperature to 20°C. If a weight is hung on the swollen elastic matrix, the weight will be lifted on raising the temperature. Therefore, the cross-linked matrix can convert thermal energy into motion with the performance of mechanical work. This is thermomechanical transduction [8].

The Matrix Can Be a Thermally Controlled Superabsorbent: As the poly(VPGVG) in the coacervate state is more dense than water, it forms a viscoelastic layer on the bottom of the container. When the temperature is lowered, it dissolves and becomes molecularly dispersed throughout the volume of the container whatever its size. When the thermally contracted elastic matrix, X^{20}-poly(VPGVG) at 37°C or above, is cooled to 20°C or less, it swells to ten times the contracted volume. If the cross-links were fewer and the chains as long as necessary, it could swell to even greater volumes. Thus, the elastic matrix can be a thermally controlled superabsorbent.

Versatility of Composition and a Hydrophobicity Scale: Starting with this basic material, by design and syntheses, the parent bioelastic material can be altered to exhibit a wide range of physical properties with many potential applications. With properly chosen amino acid substitutions, the temperature of the inverse temperature transition, which is a hydrophobic folding and assembly transition, can be lowered, if the substitute residue is more hydrophobic, or it can be raised, if the substitute residue is less hydrophobic. The general formula for such substitutions is poly[f_V(VPGVG),f_X(VPGXG)] where f_V and f_X are mole fractions with $f_V + f_X = 1$ and X is any of the naturally occurring amino acids or chemical modifications thereof. A complete hydrophobicity scale of all of the naturally occurring amino acids and of ionized states and of chemical modifications thereof has thus been developed [8-10].

Accordingly, a wide range of compositions are available for adding specific functional capacities, and the transition temperature can be placed as desired within the range available to aqueous media.

Matrices for the Conversion of Chemical Energy Into Motion (Or a Chemically-Controlled Superabsorbent): When the matrix contains even a very few (2 to 4 per 100 residues) amino acid residues with ionizable side chains, increasing the degree of ionization can drive unfolding and decreasing ionization can drive folding (contraction). The matrix has thus been designed to convert chemical energy into the motion of contraction [11]. Also, of course, one can control the functional superabsorbency of such a matrix. Furthermore, because of the present level of understanding of the forces involved, it has been shown that proper design at nanometric dimensions can dramatically change the pKa of an amino acid side chain. For example, the pKa of a Glu residue has been shifted, by design, from 4.3 to 8.1 and the titration curve is relatively steep (positively cooperative) [12]. This means that an elastic matrix can be designed to swell or contract over any chosen, small (less than one pH unit) pH range.

A Change in Concentration of Salts or Organic Solutes Can Be the Chemical Energy to Drive Contraction: Increasing salt concentrations such as NaCl, NaBr, Na_2CO_3, Na_3PO_4 (in order of potency) can lower the temperature of the folding and assembly transition [13]. Accordingly, if the transition temperature were just above 37°C, addition of any of these salts will lower the transition temperature below 37°C and contraction will occur. The effect of organic solutes is more varied; a very small amount of sodium dodecyl sulfate will raise the transition temperature; guanidine hydrochloride and urea do so in order of decreasing potency. Ethylene glycol, glycerol, trifluoroethanol and dimethysulfoxide (at higher concentration) in order of increasing potency lower the temperature of the folding transition [13].

Introduction of Enzyme Specificity: Enzyme sites can be introduced into the sequence. When the cyclic AMP dependent protein kinase site of lysozyme [14], Arg-Gly-Tyr-Ser-Leu-Gly, i.e., RGYSLG, is introduced into the polypentapeptide, poly[30(IPGVG),(RGYSLG)] where I = Ile, the serine can be phosphorylated by the protein kinase and intestinal alkaline phosphatase can remove the phosphate [15-16]. Serine phosphorylation has a tremendous effect; the phosphate is more potent in raising the temperature of the folding transition than any other known chemical modification [17]. When at the correct intermediate temperature, one phosphate in 300 residues can completely unfold the protein-based polymer. Besides showing the dramatic effect of phosphorylation on hydrophobic folding, this result demonstrates the principle that reactive enzyme sites can be introduced such that the specificity of enzyme reactions can be used to drive between folded and unfolded states.

Introduction of cell Attachment Sites: As will be discussed in more detail below, the elastic, transparent matrix of Figure 1 does not support cell attachment, but addition of a cell attachment sequence such as GRGDSP converts the matrix to an excellent substrate for cell adhesion and for providing for cell growth to confluence.

BIOCOMPATIBILITY

From this brief general background, it is hoped that the depth of knowledge of this system is apparent and that the breadth of potential applications is also apparent given the properties outlined. For medical applications, however, biocompatibility must be demonstrated and this has been done for poly(VPGVG) and its γ-irradiation cross-linked elastic matrix, X^{20}-poly(VPGVG). The series of tests that have been run by the North American Science Associates, Inc. (NAmSA®) and the favorable results are given in Table I. Quoting from the abstract of the publication that reviewed the results

"Thus, this new elastomeric polypeptide biomaterial exhibits an extraordinary biocompatibility" [18].

MEDICAL APPLICATIONS UNDER DEVELOPMENT

Given the properties of the bioelastic materials noted above, a number of medical applications that are now under development can be mentioned. First there are several animal models being used to determine the efficacy of the bioelastic materials as barriers in the prevention of unwanted adhesions that result as a consequence of surgical procedures and other trauma. Second, the bioelastic sheets are also being studied for their capacity to be refractory to cell attachment and then when appropriately modified to be converted to cell attachment matrices wherein the cells can attach and grow to confluence. This is in preparation for use of the materials in tissue reconstruction. And finally, in a more direct utilization of some of the unique physical properties of various forms of energy conversion, some rudimentary efforts at designing the elastic matrices for drug delivery have been initiated.

PREVENTION OF ADHESIONS

There are 30 million surgical procedures performed annually in the U. S. and an equivalent number in Europe. A serious postoperative complication in all of these is the formation of adhesions. It has been estimated that a fourth of these could be expected to have an improved outcome if a material were available to prevent adhesion formation. In developing uses for bioelastic materials, three animal models are being considered. The first is a contaminated peritoneal model using the rat for which there is now substantial data [19]. The second is a rabbit eye model for strabismus surgery in which a sleeve of the bioelastic material is placed around the superior rectus muscle in an effort to prevent the postoperative scarring that can defeat the objective of the corrective procedure [20]. And the third, for which studies are just beginning, utilizes the bioelastic material in the calf under cardiopulmonary bypass to prevent adhesion to the lung, and in the artificial heart to wrap the Dacron® vascular graft used to conduct the blood from the pulmonary artery and the aortic valve to their remnants and to cover the Dacron® velour which itself covers the outer surface of the atrial cuffs for the purpose of preventing adhesion of the left atrium to the pericardial wall and of the right atrium to the lung. The objective is to improve the usage of the total artificial heart as a bridge to transplantation.

In the Contaminated Peritoneal Model [19].

In this rat model, the abdominal wall is scraped with a scalpel until bleeding ensues; a loop of bowel is loosely secure over the injury with a loop of suture; the bowel is then punctured at the site apposition four times with a 20 gauge hypodermic needle, and the intestine is milked to effect leakage of contents and contamination of the site. In the controls, all of 29 control animals exhibited adhesions. There were two sets of test animals depending on the technique used for sterilization of the bioelastic sheets. For 30 animals, steam sterilization was used, and for 29 animals ethylene oxide gas sterilization was used. After positioning the test material between wall and intestinal loop, which positioning utilized the loop of suture loosely holding the bowel in apposition to the wall, the animal was closed. At seven days, the loop of suture was cut. At fourteen days, the animals were sacrificed and the adhesions were graded 0, 1, 2, or 3 [21]. Grade 0 was the absence of adhesion; grade 1 was a single band of adhesion comprised of omental fat between omentum and wall, which adhesion offered no resistance to separation; grade 2 was a fibrous band of adhesion between viscera and abdominal wall requiring moderate force to separate; and grade 3 was adhesion requiring sharp dissection to separate. Grades 0 and 1 are considered to be

insignificant adhesions whereas grades 2 and 3 are viewed as significant adhesions. For the gas sterilized material, 59% of the animals exhibited no adhesions; 21%, grade 1; 10%, grade 2; and 10%, grade 3. For the steam sterilized material, 40% of the animals exhibited no adhesions; 16%, grade 1; 13%, grade 2; and 30%; grade 3. Of the total of seven animals that exhibited grade 2, adhesions five were due to a tear in the matrix through which the adhesions passed. In the cases of the grade 3 adhesions, the bioelastic materials was completely encapsulated, but the bioelastic material was not adherent to the fibrous connective tissue; rather, it could be easily removed with tissue forceps.

An interesting example is shown in Figure 2 where a band of adhesion formed around the bioelastic matrix. The X^{20}-poly(VPGVG) is seen as a transparent elastic matrix after two weeks of implantation. There is no fibrous capsule of any kind that surrounds the matrix. There is no inflammation where the matrix has been in contract with the abdominal wall for two weeks. The elastic matrix has been seen to remain clear without fibrous capsule and undergraded after up to six months in the peritoneal cavity. The results of this study involving 88 animals indicate that X^{20}-poly(VPGVG) is an effective barrier against adhesion formation in this surgically-induced, contaminated wound model.

In the Strabismus Surgery Model [20].

Strabismus is a disorder in which the eyes cannot simultaneously focus on the same point. The positioning of each eye is due to four rectus muscles. In strabismus surgery a rectus muscle is detached and repositioned to improve alignment of the eyes. When adhesions occur following strabismus surgery or retinal detachment surgery involving rectus muscle, sclera of the eye or conjunctiva, eye movement can be severely restricted and misalignment can also result. The use of a number of materials has been attempted in the past with such limited success that they are now largely abandoned.

Preliminary studies have now been carried out using two formulations of the bioelastic material, X^{20}-poly(VPGVG) and X^{20}-poly[3(VPGVG),(VPGFG)] where F is phenylalanine. The model utilized the rabbit eye in which the superior rectus muscle was detached at its insertion; a small patch of sclera was removed underlying the muscle; the muscle capsule in apposition to the site of sclerectomy was removed, and the muscle was reattached at its original insertion site. In this control, the sclera and the muscle were tightly adhered at one week. When sleeves of X^{20}-poly(VPGVG) were used in three animals, neither inflammation nor adhesion occurred. When sleeves of X^{20}-poly[3(VPGVG),(VPGFG)] were used, no inflammation occurred but a glistening fibrous capsule formed around the sleeve which could limit eye movement. The improvement desired for the use of X^{20}-poly(VPGVG) would be to have a material which would degrade and disappear. Work in this direction is underway as will be noted below in the drug delivery application.

In the Total Artificial Heart as a Bridge to Transplantation.

In 1989 more than 400,000 open-heart procedures were performed in the United States with a substantial percentage of these being reoperations necessitated by either progression of the coronary artery disease, graft stenoses or occlusion. These secondary procedures involve far greater risk in large part due to the formation of postoperative adhesions [22].

One of the major contributing factors in the development of adhesions following open-heart surgery results from the inability to close the pericardium without compromising the procedure due to increased heart size resulting from

cardiopulmonary bypass(CPB)-induced distention. When the native pericardium is not closed, adhesion formation between the epicardium and sternum can be so massive that reoperation is extremely difficult and with significant risk of rupturing the heart.

With the increasing number of patients requiring reoperation and with the hope that total artificial hearts eventually will allow patients to survive until natural hearts become available, there is significant need for the development of a pericardial substitute which will allow isolation of the heart and prevent adhesion formation.

Structurally, the pericardium is a sac of dense connective tissue surrounding the heart. In addition to the heart, it contains the roots of the great arteries and veins. The fibrous pericardium is lined on its inner surface by serous pericardium which consists of parietal and visceral layers. Between the two layers a narrow space, the pericardial cavity, contains a thin film of serous fluid. The two layers slide freely against each other and are continuous where the great vessels pierce the fibrous pericardium. Histologically the serous pericardium consists of another layer of flattened mesothelial cells resting on a layer of connective tissue. These mesothelial cells produce pericardial fluid which is passed into the pericardial cavity.

A number of different types of materials have been tested for use as pericardial substitutes. These include, silicone membranes, polyurethane, fascia lata, polytetraflouroethylene (Gore-Tex) patches, bovine and porcine xenographs treated with glutaraldehyde, siliconized Dacron® and dura mater [22].

Although some materials appear to have been successful in preventing adhesions in animal models where the artificial pericardial material was implanted using thoracotomy or sternotomy without cardiopulmonary bypass, the results on humans have been far less successful. A possible explanation for the difference in the results from animal experiments and human clinical trials may be due to the fact that the animal experiments did not involve cardiopulmonary bypass (CPB). Evidence indicates that CPB itself causes damage to the pericardium and epicardium in humans which may impair the fibrinolytic activity of native pericardium [22].

There is a keen awareness of the necessity and need for a material that could be used in all surgeries to minimize the amount of adhesions and fibrous connective tissue (scarring) to avoid the adhesions which induce complications such as bowel obstruction, and constrictive pericarditis, the severity of hemorrhage in redo operations, and the horrors of severing previously implanted coronary artery vascular grafts. Experiences of Olsen and colleagues in developing the total artificial heart led them to look at some membranes that might be used to eliminate the adhesions and facilitate reoperations such as in replacement of the devices as well as explantation preceding cardiac transplantation. Smooth glutaraldehyde-treated pericardial allografts were used, without success [23].

In studies just now getting underway, the Utah team is to test X^{20}-poly(VPGVG) and other formulations at three sites in the calf under cardiopulmonary bypass. Adhesion problems at these sites have been considered one of the primary factors in relating results from animal studies to the human experience, according to Gabbay [22]. The plan is to place a sheet of the test materials over the lung and beneath the pleural suture line after the excision of the fifth rib in the right thorax, by tacking the four corners of the sheet, fixing the materials between the pleural suture line and the lung, an area ubiquitous with adhesions following total artificial heart implantation in the calf.

The second mode will be to wrap the Dacron® vascular graft used to conduct the blood from both the pulmonary artery and the aortic valve to the remnant aorta and pulmonary artery.

This vascular graft comes in contact with the lung, where large adhesions form, making explant of the device very difficult. The third test area will be to cover the Dacron® velour covering that is placed onto the exterior of the artificial atrial cuffs, that also adhere to the surrounding tissue, both to the pericardial wall on the left atrium and to the lung on the right atrium. These membranes will be placed in animals designed to survive from one to eight months, at which time the animal will carefully be autopsied, and the degree of scarring and adhesions carefully evaluated and compared to the scarring in the control calf.

These studies to enhance the use of the total artificial heart as a bridge to transplantation add an important dimension to the use of bioelastic materials in the prevention of adhesions and one in which the rate of degradation of the material need not be increased.

DEVELOPMENT OF ELASTIC MATRICES FOR CELL ATTACHMENT AS A STEP TOWARD TISSUE RECONSTRUCTION

It is becoming increasingly apparent in order for cells to function properly in a tissue that they require proper attachment to the extracellular matrix. The attachment occurs through receptors in the cell membrane called integrins which bind to specific peptide sequences within proteins comprising the extracellular matrix. The most celebrated cell-attachment sequence is the RGD-sequence of fibronectin to which fibroblasts and other cells attach [24-28]. The role of the attachment is in part to transmit tensional forces to which a tissue is subjected through the cell membrane to intracellular components. The changed stresses on the intracellular components such as the cytoskeleton result in chemical signals for the cell to function in such a way as to reinforce the tissue to withstand the applied stresses. The chemical responses within the cell include "ion transport, release of chemical second messengers, protein synthesis, secretion, and even expression of specific genes" [29]. Evidence of this was provided some years ago by Glagov and colleagues in which it was found that vascular wall cells when attached to vascular elastic lamina would turn on production of extracellular macromolecules in response to cyclic stretching in an effort to rebuild the natural vessel wall [30-32]. This has more recently been demonstrated in an elastic vascular prosthesis by the Dutch group [33,34]. The challenge for the vascular prosthesis is to have a functional elastic vessel that would serve as a temporary functional scaffolding that would degrade as the natural vessels were regenerated.

As will be shown below, functional cell attachment sequences can be introduced into the elastomeric polypeptide to form matrices that provide for cell attachment. It may also be noted that the physical properties demonstrated in these matrices by virtue of their capacity to undergo inverse temperature transitions demonstrate a likely mechanism whereby stretching of protein could result in chemical responses such as dephosphorylation/phosphorylation, pH changes, etc. For example, these matrices when appropriately designed exhibit stretch-induced pKa changes, i.e., stretch-induced changes in pH [35]. To our knowledge, only the bioelastic materials containing cell attachment sequences can provide for both cell attachment and the dynamic elastic response necessary to signal the cell to function in a manner appropriate to the tissue of residence. These properties unique to bioelastic materials can be expected to secure a role for elastomeric polypeptide matrices in tissue reconstruction and engineering.

Cell Adhesion in the Absence and Presence of Serum [36,37].

When synthesizing the elastomeric polypeptide, it is often an advantage to polymerize the GVGVP permutation with Pro at the carboxyl terminus rather than VPGVG with Gly at the terminus. This, of course, gives the same polymers differing only in the particular residues that begin and end the several hundred residue sequence. Thus, a

polymer containing 20 GVGVP pentamers to one cell attachment sequence GRGDSP would be written poly[20(GVGVP),(GRGDSP)] and the cross-linked matrix X^{20}-poly(GVGVP) is essentially identical to X^{20}-poly(VPGVG).

First to be discussed as a baseline or control for the cell attachment studies are X^{20}-poly(GVGVP) and a structurally related elastomeric X^{20}-poly(GGAP) [37]. As seen in Figure 3, human umbilical vein endothelial cells(HUVEC) attach to neither elastic matrix when the appropriate culture medium contains 0.1% bovine serum albumin(BSA). When the culture medium contains 20% fetal bovine serum(FBS), there is poor cell adherence to X^{20}-poly(GVGVP) without capacity for growth to confluence but there are still no cells whatever adherent to X^{20}-poly(GGAP). When ligamentum nuchae fibroblasts(LNF) are used as in Figure 4 again only poor adherence is observed with X^{20}-poly(GVGVP). This is interpreted to suggest that some serum proteins can weakly adsorb on the surface of X^{20}-poly(GVGVP) but not on the less-hydrophobic X^{20}-poly(GGAP). One implication of these results is that X^{20}-poly(GGAP) could possibly provide an even better barrier for adhesion prevention than X^{20}-poly(GVGVP) for those surgical procedures where there is much bleeding.

When using the X^{20}-poly[20(GVGVP),(GRGDSP)] and low-density platings of 1×10^4 cells/ml, the cells are seen to have attached and grown to confluency at three days (See Figure 5) [36]. These bioelastic surfaces appear to be equivalent to fibronectin coated surfaces in their ability to support cell attachment and growth to confluency.

Cell Receptor Specificity.

In the process of further characterizing the X^{20}-poly[20(GVGVP),(GRGDSP) cell attachment matrix, the interaction with blood platelets was considered. The blood platelets did appear on the surface, but did not spread or show signs of being activated, and could be rinsed off. Since platelets have the fibronectin receptor, this prompted characterization of the receptor that was responsible for fibroblast and endothelial cell attachment. As shown in Figure 6, using a series of cell adhesion inhibiting peptides [39], it is not the cell membrane receptor that binds to fibronectin that is responsible for the attachment of human umbilical vein endothelial cells(HUVEC) to the bioelastic matrix but rather the vitronectin receptor [38] in which the sequence recognized is RGDV [40]. The same is the case for ligamentum nuchae fibroblasts. This raises the attractive possibility for a vascular graft material which would encourage endothelial cell coverage of the intima without platelet activation.

A Step Toward an Artificial Pericardium.

The capacity to design elastomeric matrices that support cell attachment and growth and possibly remodeling of the matrix, the interest in developing a material to prevent adhesions following open-heart surgery, and the associated special problems of cardiopulmonary bypass-induced distention of the heart with the resultant inability to close the pericardium, combine to raise the possibility of developing an artificial pericardium. The elastic artificial pericardium would provide the added area required to cover the distended heart; it would contain cell attachment sites for mesothelial cells; and suitable fibrinolytic activity could be attached to the serosal surface. It could be designed to contract as the heart size returned to normal, and it is anticipated that it could be remodeled into natural pericardium.

There are, of course, numerous tissue reconstruction or tissue engineering applications, that may be considered, and these will be pursued as the stimulation of interested parties, research and development monies and time allow. It is also apparent

that these bioelastic matrices to which cells can attach, being both optically transparent and stretchable, would have many uses in basic research on cellular functions.

DEVELOPMENT OF DRUG DELIVERY DEVICES

Current Perspective on Drug Delivery. There is an ongoing interest in the development of materials for use in drug delivery systems both in the clinical and pharmaceutical communities. The clinical interest stems from the need for delivery stems for thereapeutic agents which can improve both the safety and efficacy of existing therapeutic agents, increase control over release patterns, provide for diversity in the use of existing agents and provide a means of delivering complex agents such as proteins in their active state. The pharmaceutical industries interest stems from an interest in gaining broader uses for existing agents.

Korsmeyer and Peppas [41] developed a classification system for controlled-release systems based on the mechanism which controls the release of the agent. The most common mechanism is Diffusion-controlled of which there are two types, (1) the Reservoir type in which the bioactive agent forms a core surrounded by an inert diffusion barrier and (2) the Monolithic type in which the active agent is dispersed or dissolved in an inert polymer. The second class consists of Chemically-controlled systems which utilize bioerodible or pendant chain systems. The advantage of bioerodible (or biodegradable) systems is that the device is eventually absorbed by the body and thus need not be removed. The third class consists of the Solvent-controlled system. In this case the agent is dissolved or dispersed in a polymeric matrix through which it cannot diffuse. As solvent penetrates the matrix, the polymer swells allowing the agent contained in it to diffuse. As solvent penetrates the matrix, the polymer swells allowing the agent contained in it to diffuse through the polymer. The fourth and final class consists of the Mechanically-controlled systems in which external forces control the release of therapeutic agent.

According to Lloyd [42], properties exhibited by the ideal macromolecular drug carrier would include the following: adequate drug-loading capacity, retention of water solubility when drug-loaded, molecular weight high enough to prevent glomerular filtration, but low enough to reach all cell types, unmodified carrier not captured by adsorptive pinocytoses, a stable carrier-drug linkage in body fluids but degradable in the lysosome, slowly biodegradable in extracellular compartment or degraded in the lysosome, non-toxic, non-immunogenic and generally biocompatible.

A large number of both synthetic and natural polymers have been studied for possible application in drug delivery. According to Ranade [43], the greatest advantage of the synthetic polymers is the wide choice available. Two promising synthetic polymers which have been developed are polyvinylpyrrolidone (poly VP) and N-(2-hydroxypropyl)methacrylamide (poly HPMA), with the latter having the advantage of being easily derivatized. The natural polymers have the advantage of being biodegradable. Among the natural polymers studied are poly(lactic acid), copolymers of DL-lactic acid and glycolic acid, and copolymers of gluconic acid and α-ethyl-L-glutamate.

Bioelastic Materials Add New Dimensions to Drug Delivery [44,45].

Molecular (Bioelastic) Machines as Drug Delivery Vehicles. A molecular machine is a macromolecular construct that can convert energy from one form, or one location, to another. The living organism may be described as the synergistic integration of functionally diverse molecular machines [13]. Biology's molecular machines are proteins. The bioelastic materials are protein-based polymers that can be designed to carry out all of the energy conversions recognized in living organisms [8,13] except

light driven processes. Studies to demonstrate light-driven folding and unfolding are in progress. Several free energy conversions for which there is now data to have demonstrated the conversion or the feasibility of the conversion are given in Figure 7.

A particularly recognizable energy conversion is the conversion of chemical energy into useful mechanical work. This is the chemomechanical transduction arrow of Figure 7. When the chemical energy is provided by the chemical alteration of the diseased state, drug delivery can be the useful mechanical result. When there is a difference in the concentration of chemical between the normal state and the diseased state, the bioelastic material could be designed to contract or to swell, either of which, depending on the vehicle design, could effect drug release. If the bioelastic materials were the envelope surrounding contents which included the drug, a chemically triggered contraction could result in expulsion. If the drug were in a monolith slab, a chemically-induced swelling could result in diffusional release. This integration of chemical-control and mechanical-control in a single device adds a new dimension to the Korsmeyer and Peppas classifications [41].

All of the arrows ending at the mechanical apex of Figure 7 originate at energy sources that could be used for mechanically-effected drug delivery. The free energy sources involve as the intensive variable a change in pressure, a change in temperature, a change in chemical potential (i.e., a change in the concentration of a chemical), and a change in electrochemical potential (i.e., a change in the oxidative state of a redox moiety attached to the protein-based polymer). At the chemical apex alone, there is all of the diversity of the chemical changes that was noted in the introduction. The changes could be intrinsic to the polymer as in a change in the degree of ionization, a change in the state of phosphorylation, etc. The changes could be extrinsic to the polymer as in the changes in concentrations of salts and organic solutes. At the electrical apex there are all of the chemical moieties that could function as redox couples, and these could be prosthetic groups such as the nicotinamides or flavines that can be oxidized or reduced by a relevant enzymatic activity. Thus, there can be designed many bioelastic constructs that could utilize many unique and relevant energy sources with which to achieve drug delivery. These free energy transductions are reviewed in a general article entitled "Free Energy Transduction in Polypeptides and Proteins Based on Inverse Temperature Transitions" [8]. Some of the specific constructs and the mechanochemical couplings are considered in more detail elsewhere for drug delivery [44,45]. Here, two more elements will be considered: control of the vehicle size and control of vehicle degradation.

Control of Vehicle Size.

Lloyd [42] in listing the properties for an ideal macromolecular drug carrier included the property of molecular weight as high enough to prevent glomerular filtration but low enough to reach all cell types. Studying the early events of the thermally-elicited hydrophobic folding and assembly, that is, the early aggregational steps of the temperature transition, using elastic- and quasi-elastic light scattering [46,47], it has been possible to find conditions for controlling aggregate size. The particle sizes can be set depending on temperature, concentration and time with radii ranging from 50 nm to 500 nm. In particular, the light scattering evidence indicates that particles with diameters centered at 200 nm can be stabilized. Now, if these could be fixed by cross-linking at such a size; they could be loaded with drug by thermal swelling followed by a contraction to trap the drug dispersed within the nanosphere. Such a size could be expected to be too large to pass through normal vasculature but would be small enough to pass through diseased vasculature. If the correct ionizable function could be designed within the particle such that the particle could be induced to swell in response to a change in pH due to the diseased state, for example, then the particle could be expected to escape the vasculature into the diseased tissue and to swell for a more rapid release of

the drug at those sites with the altered pH.

With the proper design of the bioelastic material, it appears possible to set the pKa of a functional side chain as desired. For example, by properly designing the protein-based polymer it has been possible to shift the pKa of a Glu residue from 4.3 to 8.1 [12]. The sequence that achieves this is poly[GEGFP GVGVP GVGFP GFGFP GVGVP GVGFP] where E is Glu and F is Phe. Related studies are underway to determine the magnitude of shifts that may be achieved for Asp(D), Lys(K), His(H) and Tyr(Y). In addition, it is relevant to note, as shown in Figure 8, that the titration curves in such designs for shifting the pKa can be very steep such that a change in pH of a fraction of a pH unit can effect the change from contracted to swollen or the reverse, i.e., the change required for complete drug release. Finally, it is expected that the change in volume of such a designed nanoparticle could be a factor of ten or more.

Control of Vehicle Degradation [28].

As reflected in the reviews of Korsmeyer and Peppas [41], of Lloyd [42] and of Ranade [43], degradation, whether due to bioerosion, biodegradation, or hydrolytic breakdown, is a useful means with which to control drug delivery, and it removes the delivery vehicle. But we have seen above that free energy transduction can be used in bioelastic materials to control drug release. In addition, the swollen bioelastic matrix would be expected to degrade, as it would be more accessible to proteolytic digestion than the contracted matrices that have been used in the prevention of adhesion models discussed above.

As has been discussed elsewhere, chemical clocks can be introduced in to the bioelastic matrix that would control the rate of swelling. The chemical clock could be in the side chain as, for example, an asparagine or a glutamine whose carboxamide can convert to carboxylates with half-lives depending on the sequence in which they are found ranging from days to decades [49]. This could trigger the swelling for drug release and for removal by enzymatic degradation. The chemical clock could also be in the backbone such as when replacing a glycine residue by a glycolic acid residue. In this case, hydrolytic cleavage would result both in drug release and in polymer breakdown.

The time dependence of the breakdown of asparagine(N) to aspartic acid(D) and of the hydrolysis of the glycolic acid(G_c) residue in the polypeptide backbone can be followed by the increase in the temperature for the inverse temperature transition [48]. This is shown in Figure 9 for the protein-based polymers. I: poly(GVGVP), the control; II: poly[0.9(GVGVP), 0.1(GNGVP)]; III: poly[0.9(GVGVP), 0.1(GVG$_c$VP)]; IV: poly[0.9(GVGVP), 0.1(GNG$_c$VP)]; V: poly(VPGVG), another control; VI: poly[0.9(VPGVG), 0.1(VPG$_c$VG)], and VII: poly[0.9(VPGVG), 0.1(VPG$_c$NG)]. By the experimental design, these are hydrolytic cleavages that are occurring at the surface of the viscoleastic, coacervate state in physiological phosphate buffered saline at 37°C. From the increase, in the transition temperature, polymer III has been estimated to have a half-life to complete dissolution of 25 days. The half-life for polymer VII is much shorter, and that for polymer I, a control, is essentially infinite. Thus, with this initial demonstration of two simple chemical clocks a wide range of half-lives can be obtained.

SUMMARIZING COMMENT

This short review presents some of the medical applications that are under development. The very brief preface was to make apparent the depth of our understanding of the science underlying these materials that are capable of exhibiting inverse temperature transitions. This work is reviewed extensively elsewhere [8]. The medical applications that were discussed and the underlying science are intended to demonstrate the breadth and potential that these new materials have.

ACKNOWLEDGMENT

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FIGURE LEGENDS

Figure 1. Small sheet of 20 Mrad γ-irradiation cross-linked poly(GVGVP) designated as X^{20}-poly(GVGVP). (Note that in the polymer GVGVP is equivalent to VPGVG). This bioelastic matrix is optically transparent; it is elastic; it exhibits a thermally-driven contraction capable of doing useful mechanical work; and it exhibits salt-driven contractions capable of driving, for example, drug delivery.

Figure 2. View of the contaminated peritoneal animal model for studying the efficacy of a material to function as a barrier to prevent of postoperative adhesions. The transparent elastic sheet of X^{20}-poly(GVGVP) is seen lying against the abdominal wall and a small strip of adhesion has grown around the bioelastic matrix which was positioned between the injured abdominal wall and the apposed punctured bowel. (Note that there is no inflammation of the wall, and that the bioelastic material shows no signs of degradation). In the control animals, adhesions occurred 100% of the time in this rat model. With the bioelastic materials interposed, no adhesions occurred in 50% of the animals. Overall, for the gas sterilized matrix, either no adhesions or insignificant adhesions occurred in 80% of the 29 test animals. Submitted for publication.

Figure 3. Cell attachment studies of human umbilical vein endothelial cells(HUVEC) on either X^{20}-poly(GVGVP) or X^{20}-poly(GGAP) with either 0.1% BSA(bovine serum albumin) or 20% FBS(fetal bovine serum) added to the culture medium. Poor cell adhesion is observed on X^{20}-poly(GVGVP) only in 20% FBS. No cell adhesion is observed on X^{20}-poly(GGAP)

Figure 4. Studies on the adhesion of ligamentum nuchae fibroblasts(LNF) to either X^{20}-poly(GVGVP) or X^{20}-poly(GGAP). Again, only X^{20}-poly(GVGVP) is seen to support cell adhesion but the cells are poorly adhered and are not well-spread. Again, X^{20}-poly(GGAP) is seen to support no cell attachment whatever. This suggests that X^{20}-poly(GGAP) could be a better matrix for the prevention of postoperative adhesions even than X^{20}-poly(GVGVP) in operations where there is much bleeding.

Figure 5. Matrices of X^{20}-poly[20(GVGVP),(GRGDSP)] on which low density platings of bovine aortic endothelial cells (A) and ligamentum nuchae fibroblasts (B) are seen to have grown to confluence at three days (C) and (D), respectively. The endothelial cells are very well-adhered with the capacity in a parallel-plate flow chamber to remain attached and to align with high fluid shear stresses of 30 dynes/cm^2 (E. A. Sprague, unpublished data). Normal fluid shear stress values for arteries are 15- 20 dynes/cm^2. This figure was reproduced with permission from [36].

Figure 6. Cell attachment studies on bioelastic matrices containing the GRGDSP cell attachment sequence from fibronectin. On the right-hand side are peptides that were introduced at 1 mM concentrations in the medium to assess their capacity to compete at the cell receptor and inhibits cell adhesion [39]. The second and third columns are fibronectin and vitronectin coated tissue culture plastic, respectively. From the studies, it is concluded that the cells are binding to the GRGDSP-containing bioelastic matrix utilizing this vitronectin receptor. Reproduced with permission from [38].

Figure 7. Potential free energy transductions utilizing bioelastic materials (elastic protein-based polymers). Designed elastic protein-based polymers have been synthesized which have demonstrated thermomechanical, chemomechanical and baromechanical transduction, and which have demonstrated the feasibility for electromechanical transduction. These all utilize inverse temperature transitions exhibited by the bioelastic materials. Reproduced with permission from [8].

Figure 8. Acid base titrations (dashed curves) of a bioelastic polymer poly[0.75(GFGVP), 0.25(GEGVP)] which exhibits hydrophobicity-induced pKa shifts and polymethacrylic acid which exhibits charge-charge repulsion-induced pKa shifts. In both cases, the chemical couple is ($COOH/COO^-$). The solid curves are the theoretical shapes for the titration of a weak acid. The essential point of the data is that the titration for the bioelastic polymer is steep exhibiting positive cooperativity and requiring only a small pH change to complete the titration, whereas the titration of polymethacrylic acid is broad exhibiting negative cooperativity and requiring a much larger pH change to complete the titration. As a change in proton concentration, a ΔpH, is a change in chemical potential and as the product of the change in chemical potential times the change in moles of titrated carboxyls is the chemical energy, it is apparent that a process utilizing the chemical energy would be more efficient for the bioelastic material. Reproduced with permission from [8].

Figure 9. Use of the change in the temperature of the inverse temperature transition, T_t, with time to evaluate the rate of dissolution of bioelastic polymers I through VII as defined and discussed in the text. Reproduced with permission from [48].

TABLE I

Summary of Biological Test Results for Poly(GVGVP) and its Cross-Linked Matrix X^{20}–Poly(GVGVP)

Test	Description	Test System	Results
(1) Ames (Mutagenicity)	Determine reversion rate to wild type of histidine-dependent mutants	Salmonella typhimurium	non-mutagenic
(2) Cytoxicity	Agarose overlay determine cell death and zone of lysis	L-929 *mouse* fibroblast	non-toxic
(3) Systemic Toxicity	Evaluate acute systemic toxicity from an I.V. or I.P. injection	Mice	non-toxic
(4) Intracutaneous Toxicity	Evaluate local dermal irritant or toxic effects by injection	Rabbit	non-toxic
(5) Muscle Implantation	Effect on living muscle tissue	Rabbit	favorable
(6) I.P. Implantation	Evaluate potential systemic toxicity	Rat	favorable
(7) Systemic Antigenicity (BPAT)	Evaluate general toxicology	Guinea Pigs	non-antigenic
(8) Sensitization (Kligman Test)	Dermal sensitization potential	Guinea Pigs	non-sensitizing
(9) Pyrogenicity	Determine febrile reaction	Rabbit	non-pyrogenic
(10) Clotting Study	Whole blood clotting times	Dog	normal clotting time
(11) Hemolysis	Level of hemolysis in the blood	Rabbit blood	non-hemolytic

*Reports from North American Science Associates (NAmSA)

"Adapted with permission from Reference 18."

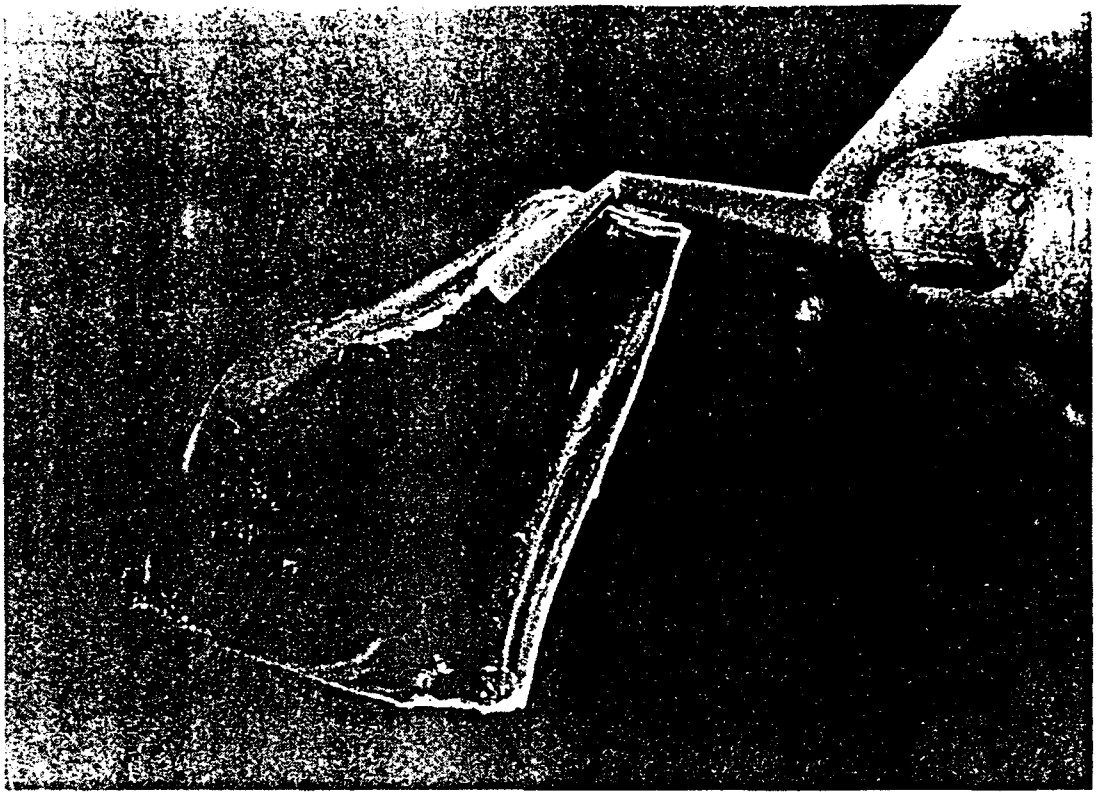


Figure 1

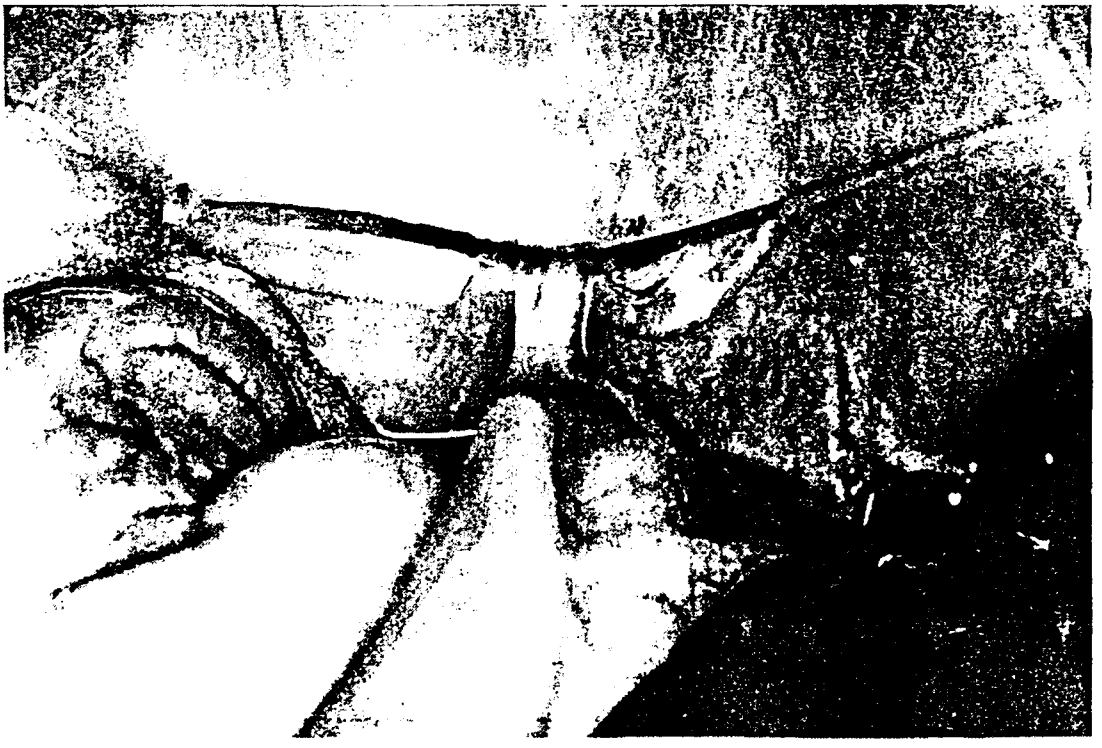


Figure 2

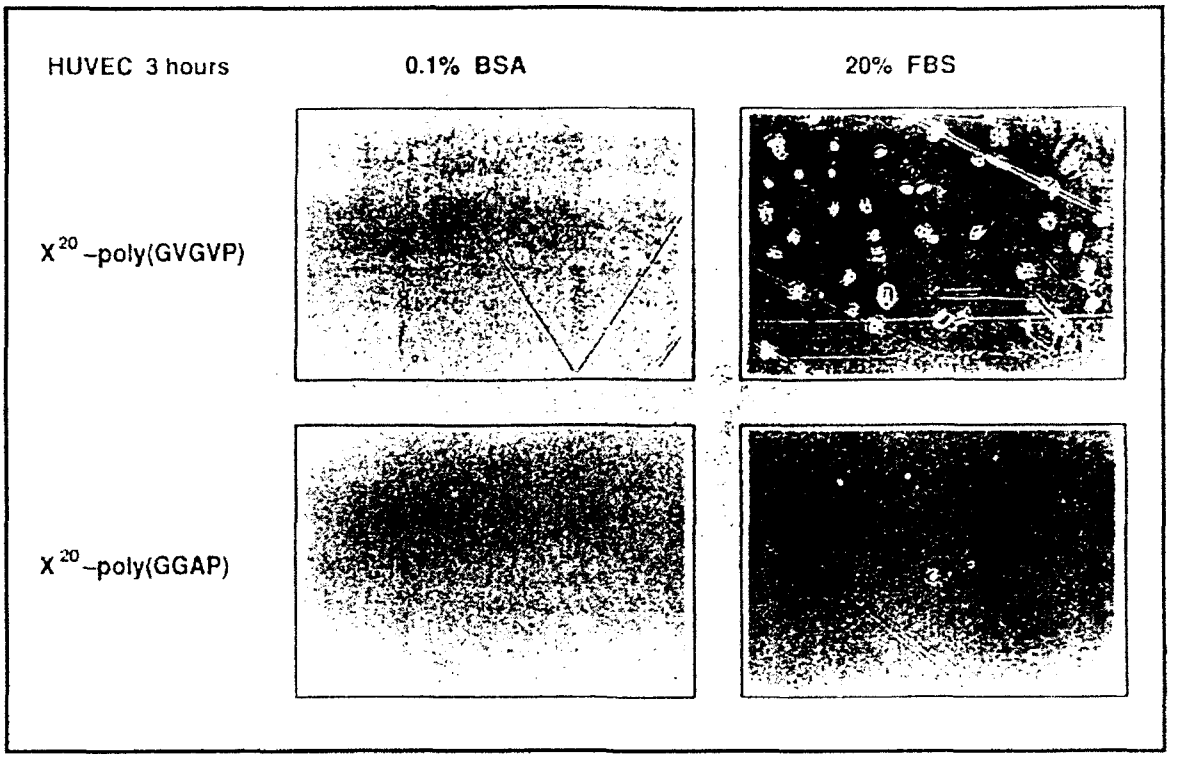


Figure 3

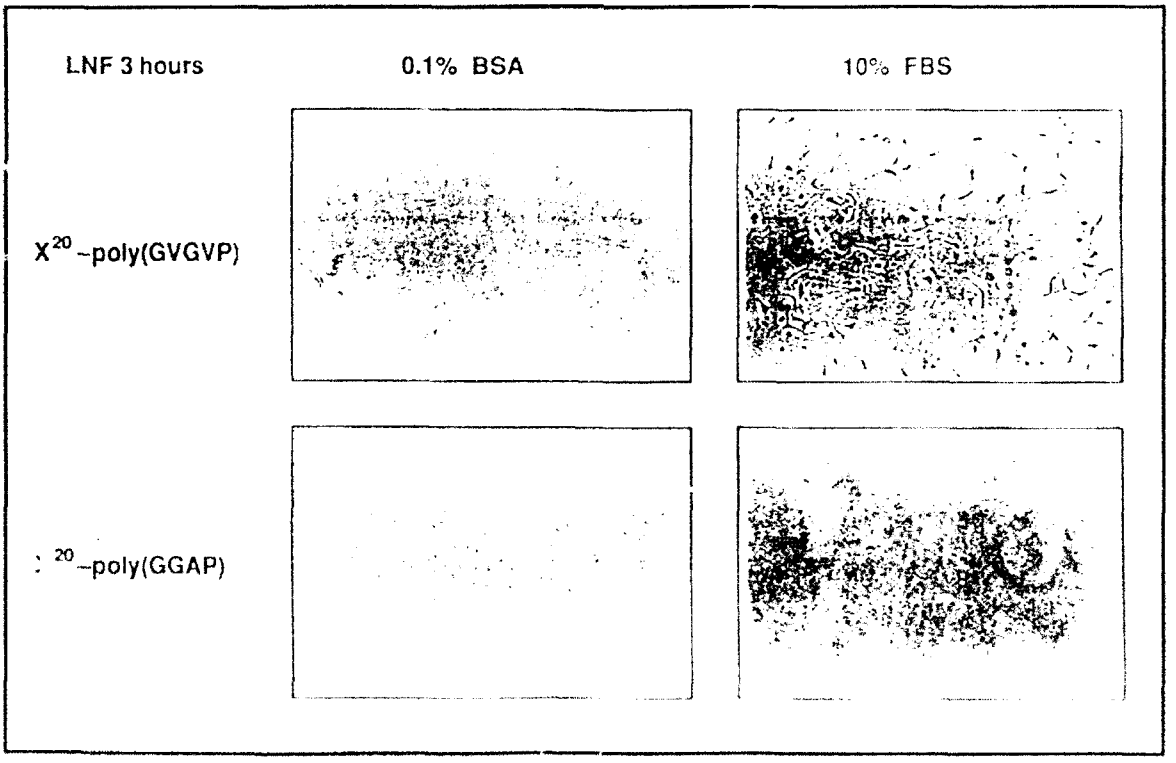


Figure 4

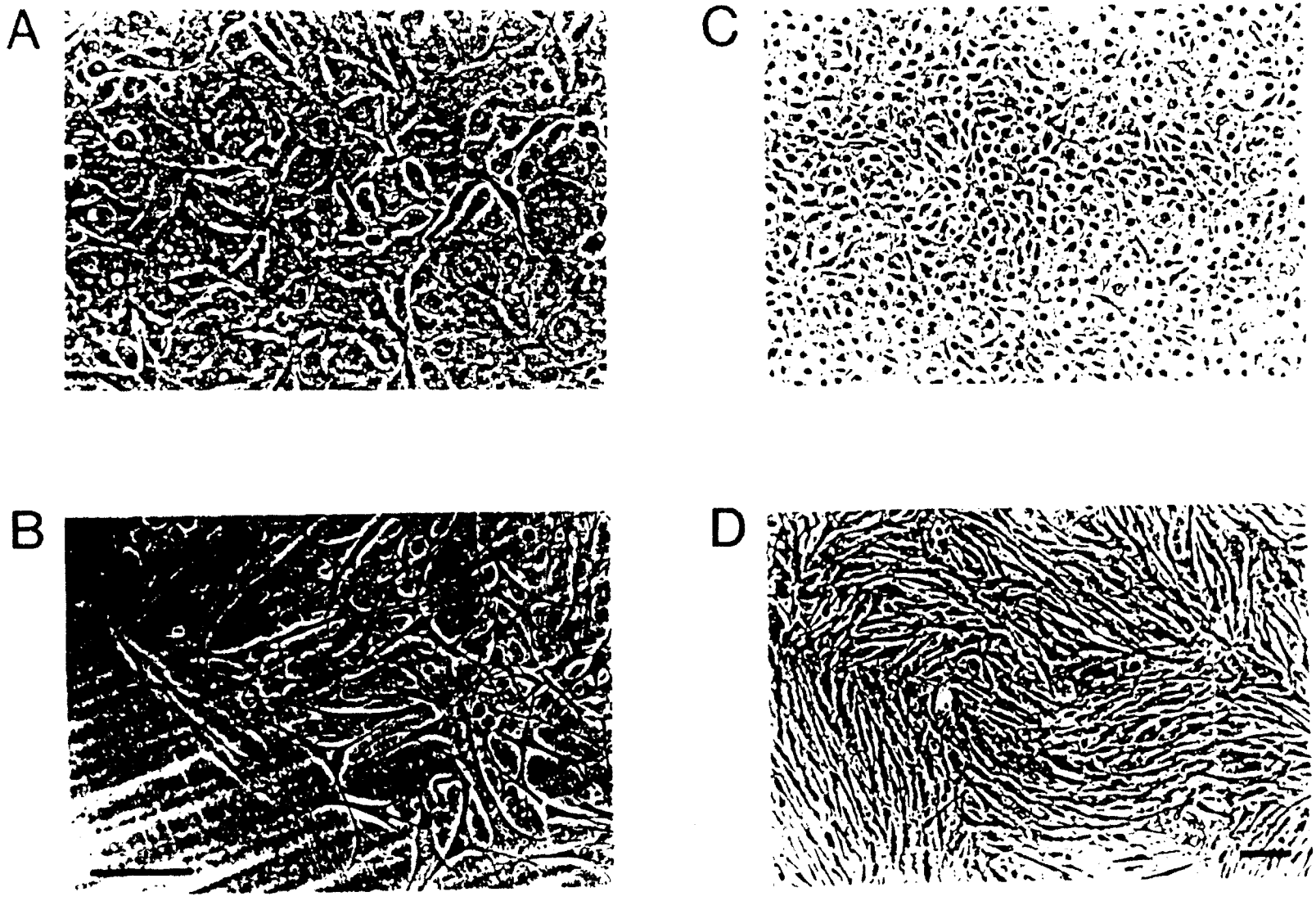


Figure 5

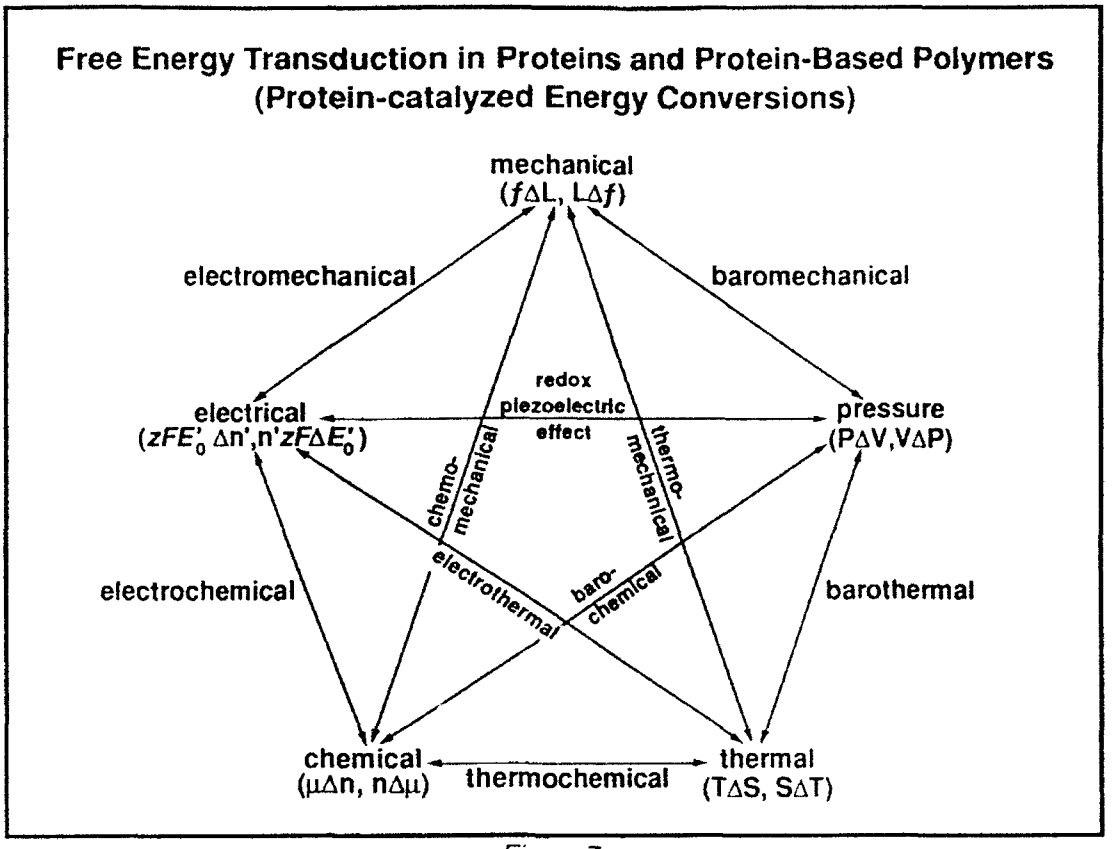


Figure 7

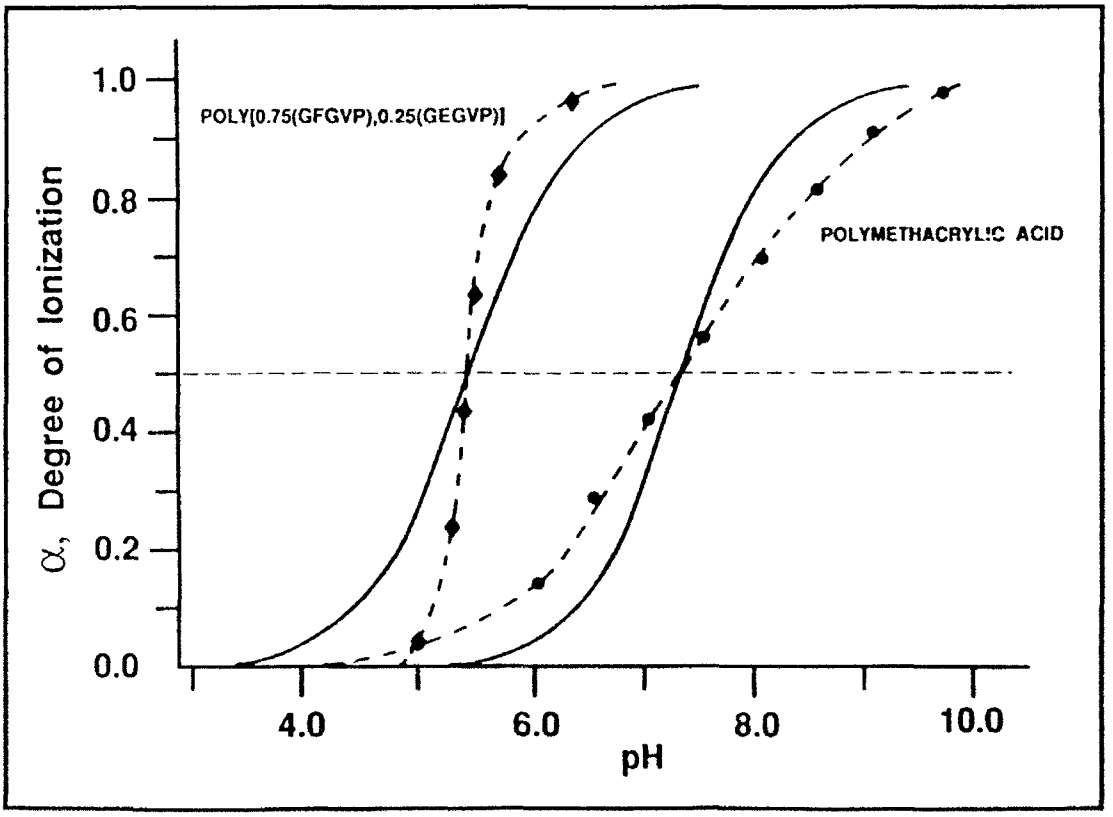


Figure 8

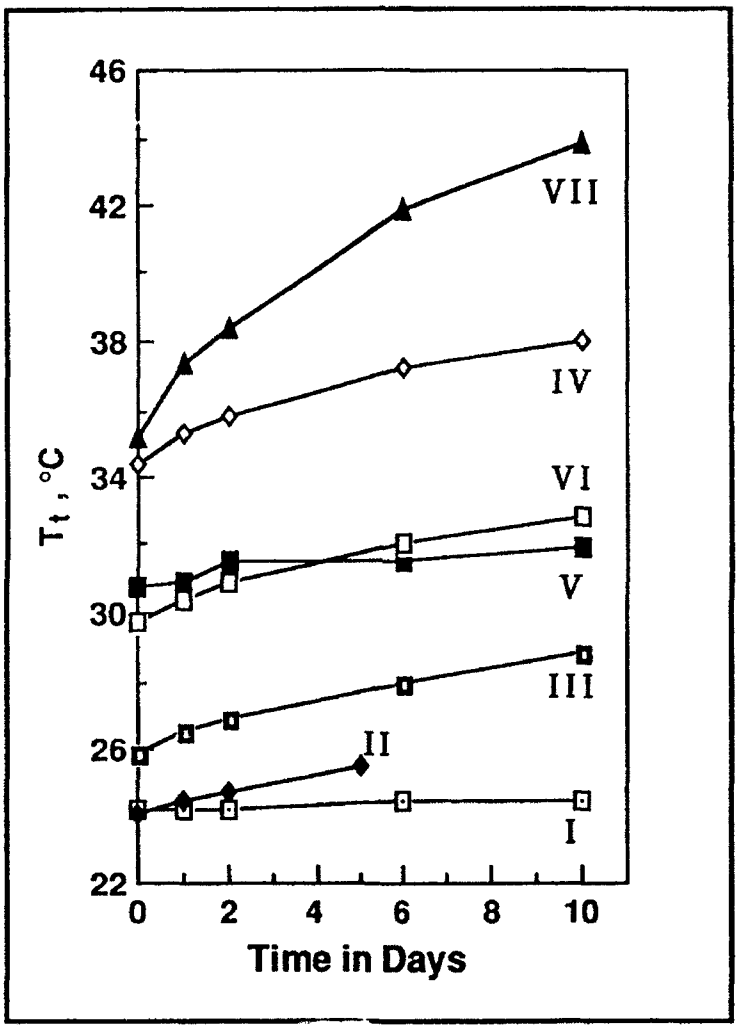


Figure 9

APPENDIX G

PROPERTIES AND PREVENTION OF ADHESIONS APPLICATIONS OF BIOELASTIC MATERIALS

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ABSTRACT

The origins, syntheses, variable composition and physical properties of bioelastic materials are discussed. The latter includes their capacity to undergo inverse temperature transitions to increased order on raising the temperature and to be designable to interconvert free energies involving the intensive variables of mechanical force, temperature, pressure, chemical potential, electrochemical potential and light.

Bioelastic materials include analogues and other chemical variations of the viscoelastic polypeptide, poly(Val-Pro-Gly-Val-Gly), and cross-linked elastomeric matrices thereof. This parent material has been shown to be remarkably biocompatible; it can be minimally modified to vary the rate of hydrolytic breakdown; it can contain enzymatically reactive sites; and it can have cell attachment sites included which promote excellent cell adhesion, spreading and growth to confluence.

One specific application is in the prevention of postoperative adhesion. There are some 30,000,000 per year surgical procedures in this country and a large portion of these would benefit if a suitable material were available for preventing adhesions. Bioelastic materials have been tested in a contaminated peritoneal model, and promising preliminary studies have been carried out in the rabbit eye model for strabismus surgery. In the peritoneal model, 90% of the 29 control animals exhibited significant adhesions; whereas, only 20% of the 29 animals using gas sterilized matrices had significant adhesions. On the basis of this data, it appears that cross-linked poly(VPGVG) is an effective physical barrier to adhesion formation in a trauma model with resulting hemorrhage and contamination.

The potential use of bioelastic materials as a pericardial substitute following the more than 400,000 open heart surgeries per year in the U.S. is under development beginning with the use of bioelastic matrices to prevent adhesions to the total artificial heart being used as a bridge to heart transplantation such that the site will be less compromised when receiving the donor heart.

BIOELASTIC MATERIALS

Origins of Bioelastic Materials

Bioelastic materials have their origins in repeating sequences of the mammalian elastic protein, elastin[1,2]. The most prominent repeating sequence occurs in bovine elastin; it can be written $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Val^4\text{-}Gly^5)_n$ where n is eleven without a single substitution. Another repeat first found in porcine elastin is $(Val^1\text{-}Pro^2\text{-}Gly^3\text{-}Gly^4)_n$ but this repeat has not been found to occur with n greater than 2 without substitution[3]. High polymers of both of these repeats, written as poly(VPGVG) and poly(VPGG) or equivalently as poly(GVGVP) and poly(GGVP), form viscoelastic phases in water and when γ-irradiation cross-linked form elastic matrices[4,5].

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Compositions of Bioelastic Materials

A wide range of compositions of bioelastic materials becomes possible when substitutions are carried out in a way that does not disrupt higher order structure formation and elastic function[6,7]. For the series of primary structures that have been considered in the prevention of adhesions applications the following general structural formula may be written for the polypentapeptides as

$$\text{poly}[f_V(\text{VPGVG}),f_X(\text{VPGXG}),f_I(\text{IPGVG})] \quad [1]$$

and for the polytetrapeptides as

$$\text{poly}[f_V(\text{VPGG}),f_X(\text{XPGG})] \quad [2]$$

where the f_i are mole fractions such that in each formula the sum of f_i is equal to one and where V = Val, P = Pro, G = Gly, I = Ile and X can be any naturally occurring amino acid or a chemical modification thereof.

The compositions may be further modified to contain enzymatically reactive sites or cell attachment sites. An example of the former is poly[30(IPGVG)(RGYSLG)] where (RGYSLG), i.e., Arg-Gly-Tyr-Ser-Leu-Gly, is a specific kinase site wherein a cardiac cyclic AMP dependent kinase can phosphorylate the Ser residue and the phosphate can be removed by intestinal alkaline phosphatase[8]. An example of inclusion of a cell attachment site is poly[40(GVGVP),(GRGDSP)] where (GRGDSP), i.e., Gly-Arg-Gly-Asp-Ser-Pro, is a cell attachment sequence from fibronectin. Whereas in a standard culture medium cells do not adhere to the matrix comprised of poly(GVGVP), they do adhere, spread and grow to confluence when GRGDSP is within the elastic matrix[9].

A further modification could be the introduction of a site for proteolytic cleavage by enzymes in the milieu of interest or by enzymes doped in the matrix for the purpose of controlling rate of degradation. The matrices can be used for releasing therapeutic agents whether being employed in the prevention of adhesions or in other drug delivery contexts. Also, the chemical synthesis wherein glycolic acid residues, Glc, replaced either of the Gly residues can provide a means of controlling rate of degradation for removal and/or for release of drugs[10].

Preparation of Bioelastic Matrices

Polymers based on the repeating elastin sequences and their amino acid analogues can be synthesized microbially by using genetic engineering[11] and they can be synthesized chemically by using classical solution and/or solid phase peptide synthesis methods or by using a combination of microbial and chemical means[11,12]. For poly(GVGVP) an estimate of the cost for large scale chemical synthesis is of the order of $20.00/gram and for large scale microbial synthesis of less than $1.00/gram.

For many applications elastomeric sheets or matrices are desired. While there are many chemical and enzymatic means of achieving cross-linking to form elastic matrices, a particularly convenient method is by γ-irradiation. An effective dose is 20 Mrads with the resulting elastic matrix being designated for example as X^{20}-poly(GVGVP). One gram of poly(GVGVP) can result in a matrix 7 cm x 7 cm x 0.4 mm when contracted and 15 cm x 15 cm x 1 cm when swollen in water. Interestingly, for protein-based polymers of Formula [1] above, nuclear magnetic resonance (even using nitrogen-15 and carbon-13 enrichment)[13,14] and amino acid analyses (in preparation) before and after 20 Mrad γ-irradiation cross-linking indicate amino acid destruction to be below detectable levels.

Physical Properties of Bioelastic Materials

Inverse Temperature Transitions: Protein-based polymers of Formulae [1] and [2] as well as a number of other compositions such as poly(APGVGV) and poly(VPGFGVGAG) are

soluble in water at low enough temperatures but on raising the temperature they self-associate with clear examples of unambiguous increase in order. This increase in order on increasing the temperature is called an inverse temperature transition[15].

T_t-based Hydrophobicity Scale: On introduction of a more hydrophobic residue, e.g., Val → Ile, the temperature of the transition, T_t, is lowered and on introduction of a less hydrophobic residue, e.g., Val → Ala, the temperature of the transition, T_t, is raised. In fact, a hydrophobicity scale has been developed for all of the naturally occurring amino acid residues, their different states of ionization when relevant, chemical modifications, and biologically relevant prosthetic groups[7]. This is referred to as the T_t-based hydrophobicity scale; it provides the molecular engineer with the capacity to design materials of desired properties.

Elasticity: The elastic (Young's) modulus for X^{20}-poly(GVGVP) is about 1 x 10^6 dynes/cm^2 (10^5 Newtons/m^2) with little or no hysteresis and with extensions of up to 200% having been observed. The elastic modulus is proportional to the square of the γ-irradiation dose; for example, a doubling of the dose quadruples the elastic modulus. Depending on the composition the elastic modulus for a 20 Mrad dose can vary from 10^5 dynes/cm^2 to 10^9 dynes/cm^2. When determining the temperature, T, dependence of force, f, at fixed length, a plot of ln (f/T) versus T approximates a zero slope when above the transition temperature range such that by classical arguments X^{20}-poly(GVGVP) is a dominantly entropic (ideal) elastomer[6] as is natural elastin with the potential for the remarkable durability exhibited by natural elastin which appears to be capable of sustaining billions of demanding stretch/relaxation cycles in the aortic arch.

Thermomechanical Transduction: For those compositions such as Formulae [1] and [2] that form viscoelastic phases above the temperature, T_t, of the inverse temperature transition, they may be shaped as desired, e.g., as sheets, and γ-irradiation cross-linked to form the elastic matrices described above. These matrices exhibit reversible contraction and relaxation, i.e., de-swelling and swelling, on passing through the inverse temperature transition. On raising the temperature from below to above T_t the elastic matrices can contract and perform the mechanical work of lifting a weight. They can perform thermomechanical transduction[15,16].

The ΔT_t-Mechanism of Free Energy Transduction: Now instead of raising the temperature from below to above T_t to drive contraction, it has been shown that there are many ways to lower the value of T_t from above to below a working temperature to achieve contraction and the performance of mechanical work. This is called the ΔT_t-mechanism of free energy transduction[15,16]. Decreasing the degree of ionization of a Glu(E) residue, for example, is a dramatic means of lowering the value of T_t and addition of protons to a Glu-containing matrix has been shown isothermally to drive contraction. This is referred to as intrinsic chemo-mechanical transduction. As increasing salt (NaCl)

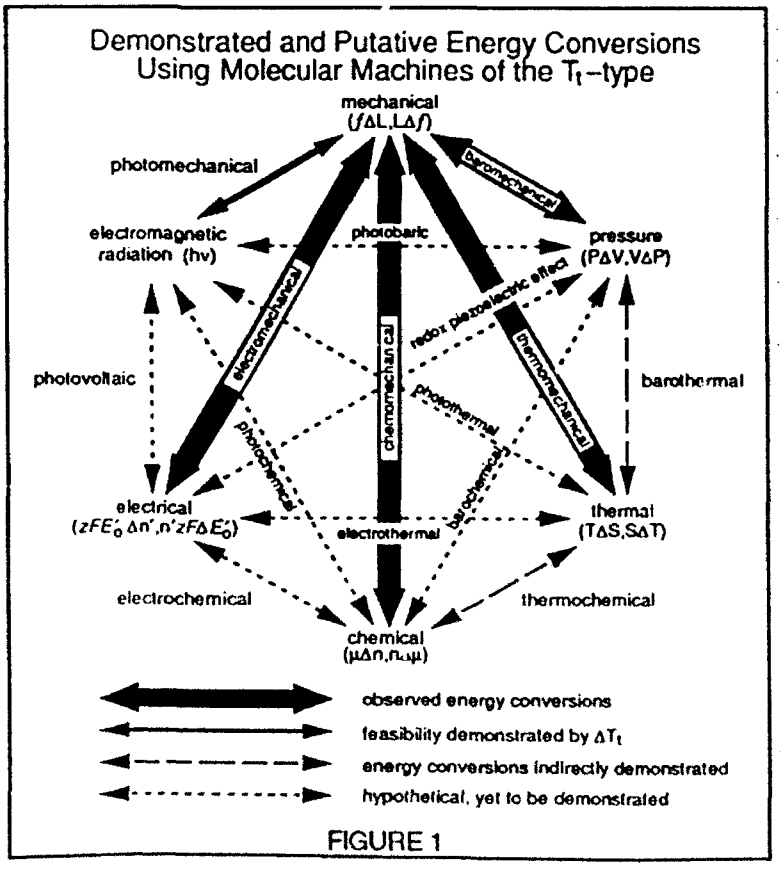


FIGURE 1

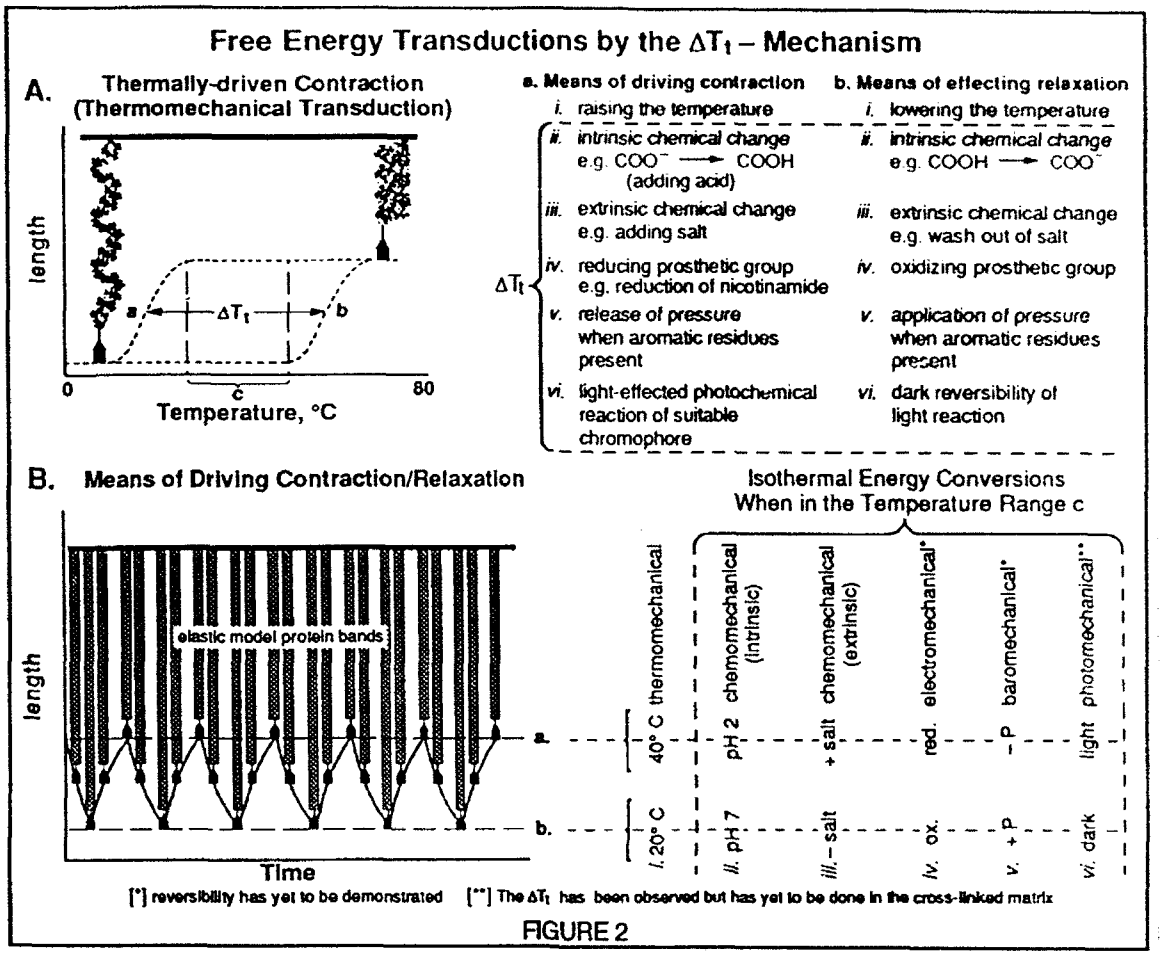


FIGURE 2

concentration lowers T_t, this too becomes a means of driving contraction referred to as extrinsic chemomechanical transduction.

Energy Conversions Possible by the ΔT_t-mechanism: In fact, the bioelastic matrices can be designed for many forms of free energy conversion involving the intensive variables of temperature, pressure, chemical potential, electrochemical potential, light and mechanical force as depicted in Figure 1. A summary of the ΔT_t-mechanism is given in Figure 2[16].

Biocompatibility of Bioelastic Materials

With the mammalian origin and nature (dynamic with a dominantly hydrophobic structure) of the bioelastic materials, it had been anticipated that these materials would be biocompatible. Following the recommendations for the set of generic biological tests required to establish biocompatibility for materials in contact with tissues, tissue fluids and blood, eleven tests were performed on poly(GVGVP) and the elastic matrix, X^{20}-poly(GVGVP). With the results following in parenthesis, these were: *(1) the Ames mutagenicity test (non-mutagenic), (2) cytotoxicity-agarose overlay (non-toxic), (3) acute systemic toxicity (non-toxic), (4) intracutaneous toxicity (non-toxic), (5) muscle implantation (favorable), (6) acute intraperitoneal toxicity (non-toxic), (7) systemic antigenicity (non-antigenic), (8) dermal sensitization—the Magnusson and Kligman maximization method (non-sensitizing), (9) pyrogenicity (non-pyrogenic), (10) Lee-White clotting study (normal clotting time), and (11) *in vitro* hemolysis test (non-hemolytic)*[17]. The result is a remarkable biocompatibility.

In addition, biocompatibility tests are underway on a member of the polytetrapeptide series, namely poly(GGAP) and X^{20}-poly(GGAP). To date the series of tests—the Ames mutagenicity test, cytotoxicity-agarose overlay, systemic toxicity, Kligman sensitization and hemolysis—also underscore good biocompatibility. Further information is available from peritoneal implants in the rat where numerous additional compositions of the Formulae [1] and [2] class of bioelastic materials all appeared to be biocompatible with X = Phe(F), Ala(A), Glu(E), and Ile(I).

Cell Attachment to Bioelastic Matrices

The bioelastic matrices—X^{20}-poly(GVGVP), X^{20}-poly(GGIP), X^{20}-poly(GGVP) and X^{20}-poly(GGAP)—do not result in cell adhesion by fibroblasts and vascular endothelial cells in appropriate cell culture media. When 10% fetal bovine serum is used in place of 0.1% bovine serum albumin, cell adhesion is observed with bovine ligamentum nuchae fibroblasts adhering better than human umbilical vein endothelial cells and with the order of decreasing cell adhesion being X^{20}-poly(GGIP) > X^{20}-poly(GGVP) > X^{20}-poly(GVGVP) but with no cell adhesion even in the presence of serum for X^{20}-poly(GGAP)[18].

On introduction of the GRGDSP cell attachment sequence[19], as in X^{20}-poly[40(GVGVP),(GRGDSP)], the bioelastic matrix presents a surface on which cells will attach and spread and grow to confluence[9]. The cells include bovine ligamentum nuchae fibroblasts, bovine aortic endothelial cells, human umbilical vein endothelial cells, and a human A375 malignant melanoma cell line[9,18,20].

Interestingly, the GRGDSP sequence as presented at the surface of this bioelastic matrix has been shown to be an attachment site for the vitronectin cell membrane receptor[20] rather than the fibronectin cell membrane receptor as might have been expected, as GRGDSP is the sequence in fibronectin that binds to the fibronectin cell membrane receptor[19]. Since blood platelets contain the fibronectin cell membrane receptor, this surface has the advantage of being very favorable for vascular endothelial cell attachment without favoring unwanted blood platelet adhesion and activation.

Another advantage of the bioelastic matrix designed for cell attachment arises because of its inherent elasticity and the capacity to vary the stiffness (the elastic modulus) of the matrix over a wide range of values from 10^5 dynes/cm^2 to 10^9 dynes/cm^2 ranging from a gelatin-like substance to a plastic-like material. Importantly, cells attached to a bioelastic matrix, as to the natural extracellular matrix, could sense deformations to which the matrix may be subjected in its role as a prosthesis, i.e., as a tissue substitute or replacement. Cells capable of sensing the tensional forces to which a tissue or a prosthesis is subjected function as mechanochemical transducers with the release of intracellular chemical signals that turn on genes for producing protein necessary for maintaining or reconstructing the extracellular matrix[21-23]. In this way a biodegradable bioelastic matrix could act as a temporary functional scaffolding which would have the potential to be remodeled into a natural tissue.

PREVENTION OF ADHESIONS APPLICATIONS

More than 30 million surgical procedures are performed annually in the U. S. with an equivalent number in Europe. In most of these adhesions, the formation of unwanted fibrous scar tissue binding tissues and organs together that should otherwise be separated, are a significant, and too often a severe, complication. An interesting example of a growing subset of these surgical procedures are the more than 400,000 open heart surgeries performed annually in the U. S. which require cardiopulmonary bypass (CPB) with the attending CPB-induced swelling of the heart which in turn commonly necessitates leaving open the pericardial sac which normally surrounds the heart. The result can be adhesion of

heart to the sternum, as well as other adhesions, with great danger of lacerating the heart on repeat sternotomy. A substantial number, approaching 20% at some centers, of the open heart procedures are reoperations with far greater risk due to the adhesions. In the abdominal cavity, post-operative and trauma-induced adhesions cause great discomfort and even intestinal blockage requiring reoperation with again increased risk in part due to adhesions obscuring the usual anatomical landmarks which guide the surgeon.

A. ninated Peritoneal Model in the Rat[25]

Bioelastic materials have been tested in an abdominal cavity model where, as depicted in Figure 3A, the abdominal wall is scraped with a scalpel until bleeding; a loop of intestine is repeatedly punctured with a hypodermic needle until bleeding and bowel contents can be extruded; and the injured contaminated intestine is held in apposition to the injured wall by a loose loop of suture accessible without reopening the cavity. At seven days the suture loop is removed and at two weeks the abdominal cavity is reopened and examined. This results in the intestine being bound to the wall by adhesions in 100% of the cases (29 animals) with adhesions being significant in 90% of the animals[25]. Seen in Figures 4A and B for these control animals and identified by the arrows are adhesions binding loop of bowel to abdominal wall.

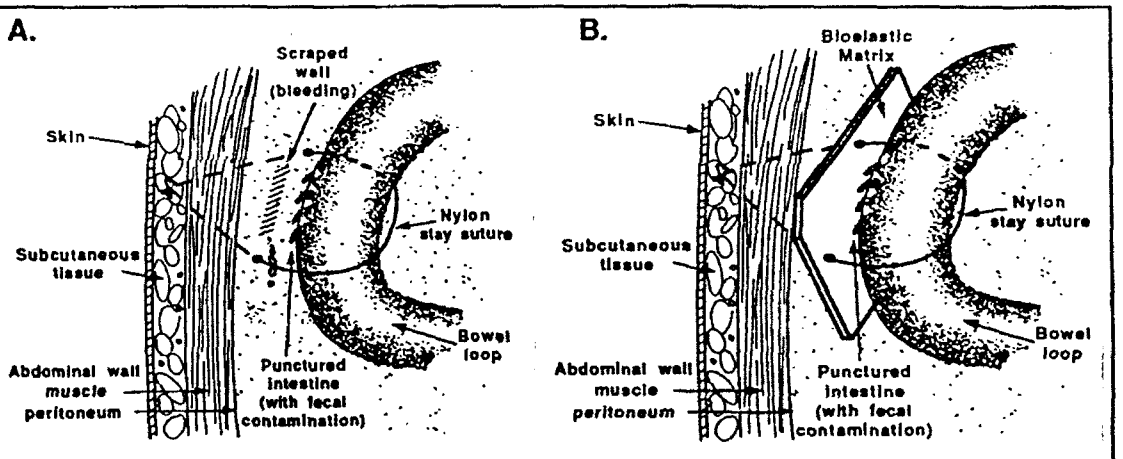


FIGURE 3

When the gas sterilized bioelastic sheet is interposed between injured wall and injured intestine as schematically shown in Figure 3B and photographed in Figure 4C, significant adhesions were prevented in 80% of 29 animals[25]. What is not apparent in the black and white print of Figure 4C is the presence of blood. In Figure 4D is the re-opened abdominal cavity showing the scarred region of the abdominal wall and the absence of any adhesions. Thus, even with the presence of blood and with frank contamination, this bioelastic matrix, X^{20}-poly(GVGVP), provided in this model an effective barrier to adhesion formation.

An instructive example is seen in Figure 4E where the vertical arrows indicate the bioelastic matrix and the horizontal arrow identifies a small loop of adhesion that has grown around the sheet of X^{20}-poly(GVGVP). The matrix is seen to have remained transparent; no fibrous coating had encapsulated the matrix in the two-week period; in fact, the matrix remains uncoated and transparent for months, seemingly ignored by the host. Through the transparent matrix it is seen that there is no sign of inflammation of the abdominal wall against which the matrix has been in contact for two weeks. Seen in Figure 4F are two bands of adhesion having grown through a break in the matrix indicated by the arrow. This occurred approximately 10% of the time, such that if this were overcome, the matrix might be expected to prevent significant adhesions some 90% of the time in this model. While other barrier materials that have been proposed for the prevention of adhesions have not

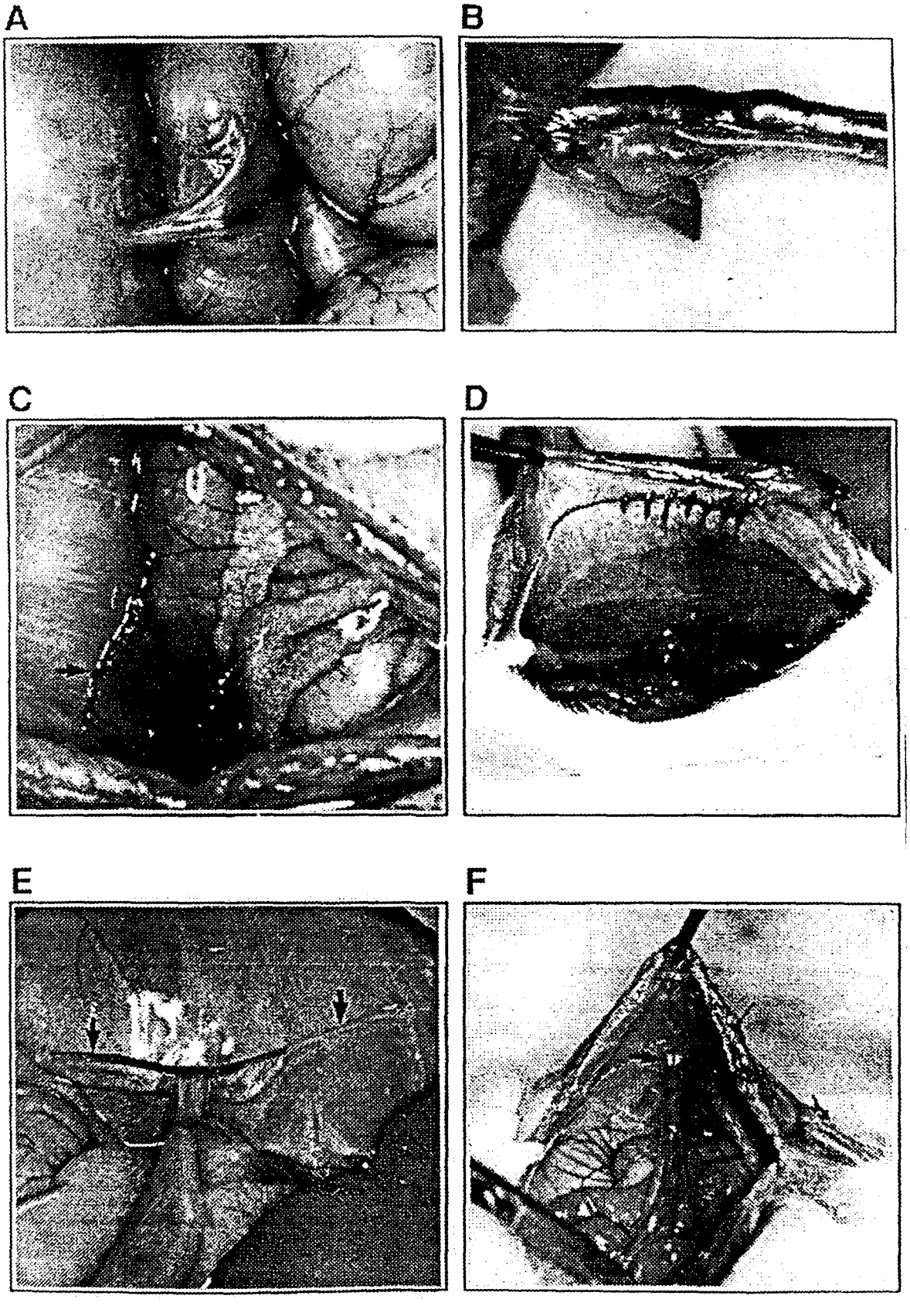


FIGURE 4

been compared in this model, this degree of efficacy has yet to be demonstrated by other materials or therapies.

These favorable findings for the X^{20}-poly(GVGVP) composition of bioelastic matrix may be obtainable by additional compositions. While there are as yet an inadequate number of animals tested for the other compositions, two were particularly promising. With four animals tested for each composition, X^{20}-poly(GGIP) was effective 100% of the time, and X^{20}-poly(GVGIP) was effective 75% of the time. Also appearing effective were X^{20}-poly[0.45(GVGVP),0.55(GAGVP)] and X^{20}-poly[0.75(GVGVP),0.25(GFGVP)]. Less effective was X^{20}-poly[0.67(GGVP),0.33(GGFP)]; with 6 animals this material was effective 50% of the time, but with the other three animals the matrix was entirely encapsulated. Exhibiting the poorest performance, but still appearing to be biocompatible, was X^{20}-poly(AVGVP) where with but three animals the material was effective 33% of the time. These additional compositions provide the opportunity for a range of physical properties; for example, they encompass the full range of elastic moduli noted above.

The serous membrane lining which covers the abdominal wall is called the parietal peritoneum, and that continuous part that is reflected over the internal organs is the visceral peritoneum. In the contaminated peritoneal model in the rat utilized above, both parietal and visceral peritoneal surfaces are injured and contaminated, and the injured sites are held in juxtaposition. This is a severe challenge to the fundamental problem of achieving repair of the serous membrane by regeneration of the mesothelial cell lining without resulting in the fibrotic response giving rise to adhesions.

Many adjunctive chemical therapies have been attempted for promoting mesothelial regeneration while limiting fibrosis resulting from surgical procedures. These include[25] heparin[26], corticosteroids[27], antihistamines[28], non-steroidal anti-inflammatory drugs[29], fibrinolytics[30], sodium carboxy methyl cellulose[31], chondroitin sulfate[32], proteolytics[33] and dextran[34,35]. Quoting from Jansen[36] in his review of "The First International Symposium for the Treatment of Post-Surgical Adhesions" held in Phoenix, Arizona September 1989, "Adjunctive therapy to promote mesothelial healing over fibrosis and formation of adhesions has a tenuous basis in the clinical practice of preventing adhesions[37-39]. Corticosteroids, antihistamines, antiprostaglandins and anticoagulants have all been used to aid healing, but properly controlled clinical studies are few and the evidence is against their use around the time of operation making a material difference to the eventual outcome."

The use of bioelastic matrices for the prevention of adhesions involves the physical barrier approach. There are many materials that have been considered as physical barriers principally as pericardial substitutes as considered further below. As recently reviewed by Gabbay[40], "These have included silicone membranes[41], polyurethane[42], fascia lata[43,44], polytetrafluoroethylene (Gore-Tex) patches[45,46], bovine and porcine pericardium xenografts (PXs) treated with glutaraldehyde[47-50], siliconized Dacron[51,52] and dura mater[53]".

Soules, et al.[54], in a comparison of the available physical barrier materials for the prevention of adhesions in the pelvic cavity, tested Gelfilm, Surgicel, Silastic, Gelfoam paste, amnion, peritoneum and omentum and concluded "The data suggest that the barrier methods actually promote the formation of adhesions...". By further designing Surgicel, specifically as a material for the prevention of adhesions, the resulting oxidized, regenerated cellulose, called Interceed (TC7), was tested in the rabbit uterine horn model[55]. Those results led to a multicenter clinical study where good surgical technique was the control with a 28% absence of adhesions and where use of Interceed and good surgical technique was the test with a 54% absence of adhesions[56]. In the Japanese multicenter clinical study for infertility and endometriosis surgery, adhesions were reduced from 76% in the controls to 41% when Interceed was used[57].

Interceed has now been approved for use in the pelvic region by the U. S. Food and Drug Administration. Even in this favorable, approved, limited anatomical use, 41% to 46% of the cases resulted in adhesion formation. In addition, Interceed is considered to promote adhesion when saturated with blood[36] and it is contra-indicated when there is

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frank infection. Thus, the need for a material or therapy to prevent adhesions remains critical to improve the outcome of surgeries in general, to prevent the chronic pain and discomfort that follow abdominal surgery, to decrease the incidence of bowel obstruction following abdominal surgery, to decrease the incidence of infertility in women due to surgical procedures in the pelvic region, and to decrease risk and improve the outcome of reoperations.

Strabismus Surgery Model in the Rabbit Eye[58]

Strabismus is a disorder of the rectus muscles of the eye which prevents both eyes from simultaneously focusing on the same point, as in crossed eyes. Corrective surgery attempts to alleviate this disorder by detachment of one of the four rectus muscles that orient the eye and reattachment in order to bring the eyes into better alignment. The complication is that the repositioned muscle can adhere (become reattached due to scarring), for example, to the old insertion site, thereby defeating attempts to achieve accurate alignment.

A number of materials—silicone[59]; a polyglactin 910 mesh[60], Supramid Extra®—have been used in the form of tubes or sleeves to improve the outcome of this surgical procedure[61,62], but these efforts have now been largely discontinued. It becomes of interest therefore to determine the possible effectiveness of bioelastic materials.

In the rabbit eye model following a modification of Sondhi's method[60], the superior rectus muscle is detached at its insertion site on the sclera; a patch of sclera 3 mm x 3 mm is removed underlying the muscle; the muscle capsule overlying the scleral injury is removed, and the muscle is reattached at its original site. At one week the muscle is tightly adherent to the scleral injury site and at eight weeks histological examination demonstrated a dense fibrovascular scar. For the test animals two compositions of bioelastic materials were used, X^{20}-poly(GVGVP) with three animals and X^{20}-poly[0.75(GVGVP),0.25(GFGVP)] with two animals. Both compositions were well tolerated by the eye with no inflammation evident after the mild inflammation of the procedure subsided within a few days. In both cases adhesion of the muscle to the overlying conjunctiva and the muscle to the sclera did not occur. A glistening fibrous capsule formed around X^{20}-poly[0.75(GVGVP),0.25(GFGVP)] within two weeks whereas no capsule formed around X^{20}-poly(GVGVP) in a two-month period. Both materials ultimately extruded through the conjunctiva of the small rabbit eye. The latter material holds promise for use in strabismus surgery, particularly if the matrix can be designed to degrade within a period of a month or two in order to prevent limiting of eye movement, and possible extrusion.

Total Artificial Heart Model in the Calf (Toward an Artificial Pericardium)

Use with the Total Artificial Heart: The properties of the bioelastic materials considered in the peritoneal model appear to be appropriate for use with the total artificial heart (TAH) as a bridge to heart transplantation. When the TAH is emplaced even for a short time, adhesions form to the surface of the device. Removal of the TAH prior to placement of the donor heart requires dissection of the adhesions which presents a compromised site for the donor heart. Work is presently underway to make sheets of X^{20}-poly(GVGVP) of appropriate size which are to be utilized by the University of Utah group under the direction of D. B. Olsen in the calf model. The periods of placement are to vary from one to six months.

Toward an Artificial Pericardium: In the more than 400,000 open heart surgeries performed per year in the U. S. , the chest is opened by splitting the sternum; the pericardial sac in which the heart resides is opened and cardiopulmonary bypass (CPB) is instituted. When the procedure, which may be the emplacement of coronary artery bypass

grafts (CABG), valve replacement or correction of congenital defects, is completed, the issue of closure is addressed. It is preferred if the pericardium can be closed, but it is often necessary to leave the pericardial sac open for several reasons[45,50,63,64]. During cardiopulmonary bypass the heart can become distended and the compression due to closure can lower the performance of the heart; compression due to closure can also compress, distort or kink the aorta-coronary bypass grafts compromising their function; the pericardium can be left open to permit drainage, and shrinkage of the pericardium may have occurred following a previous operation.

There are many conditions necessitating reoperation[50,65]: "intimal hyperplasia of saphenous vein bypass grafts, graft atherosclerosis, progression of underlying coronary artery disease[65]", prosthetic valve failure, perivalvular leakage, infection on prosthetic valves and conduits, progression of coronary artery disease necessitating repeat CABG, and congenital heart disease requiring a definitive operation following a palliative surgical procedure[50]

The increased risks on reoperation are many. Perhaps most striking are the danger of rupturing the heart as the sternum is reopened due to severe adhesions between heart and sternum resulting from having left the pericardial sac open and the danger of severing an aorta-coronary graft buried within an adhesion[66]. There is increased reoperation time, excessive bleeding due to dissection of adhesions, and degeneration of pericardial substitutes that may have been tried and adhesion of the pericardial substitute to the heart[60].

Gabbay[40] has listed desirable properties for a pericardial substitute as "(1) nonadherence to the heart and easy separability upon reoperation; (2) nonadherance to the sternum upon reoperation, so that repeat sternotomy is technically no different from the original procedure; (3) capability of mechanical attributes, and maintenance of the barrier integrity of the native pericardial sac; (4) freedom from dimensional distortion or shrinkage upon prolonged implantation; (5) convenience and technical ease of handling; (6) immunologic inertia, so as not to provoke inflammatory host response; and (7) capability of acquiring fibronolytic activity similar to nature pericardial tissue".

The consideration of bioelastic materials as a pericardial substitute presents a challenge to which these new materials are well-suited. While it would be possible to discuss bioelastic matrices in terms of each of the desired properties noted above, only three aspects will be briefly noted. One is the capacity to design bioelastic matrices to have an elastic modulus in the range exhibited by the pericardial sac; the second is the demonstrated capacity in the peritoneal and eye models noted above not to adhere to tissues undergoing repair; and the third is the capacity to introduce cell attachment sequences and to provide a matrix on which those cells can function. Thus, identification and incorporation of cell attachment sequences for the mesothelial cells that line the pericardium and for the underlying mesenchymal cells could provide for a pericardial substitute that could be remodeled to form a functional pericardium with, among other properties, a fibrinolytic activity.

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USE OF POLYPENTAPEPTIDES OF ELASTIN TO PREVENT POSTOPERATIVE ADHESIONS: EFFICACY IN A CONTAMINATED PERITONEAL MODEL

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ABSTRACT

We investigated the use of a sheet of polypentapeptide of elastin as a physical barrier to adhesion formation in a contaminated peritoneal wound model. The material can be supplied in sheets, liquid or foam. A total of 88 rats were studied with random assignment of animals to three study groups: Control (29), polypentapeptide steam sterilized (30), and polypentapeptide gas sterilized (29). Animals were anesthetized and a laparotomy conducted to reveal the cranial portion of the ileum. The abdominal wall muscle peritoneum was excoriated until hemorrhage was noted. In sham animals, there was no physical barrier placed between bowel loop and the abdominal wall. In the study groups, the polypentapeptide sheet was placed directly cver the excoriated area. The intestinal loop was then loosely secured to excoriated area with 2-0 nylon (stay suture) which was tied subcutaneously in all groups. Four puncture wounds were made with a 20-gauge hypodermic needle in the bowel that was apposed to the excoriated peritoneal musculature which allowed leakage of intestinal contents and contamination. On day 7 post surgery, the animals were anesthetized and the stay suture was removed. On day 14, all animals were sacrificed and adhesions graded. The incidence of significant adhesions was 28% for barrier group versus 90% for control animals ($p<0.05$). The results of this study indicates that the polypentapeptide of elastin sheet is an effective physical barrier in this surgically

induced contaminated wound model. Further investigation utilizing liquid and foam forms of this material are needed to completely evaluate the potential applications.

INTRODUCTION

Despite the advances in modern surgical techniques, postoperative intra-abdominal adhesions is a common sequela of pelvic and abdominal operations [1, 2]. Abdominal adhesions consist of fibrofatty tissue that interconnects loops of bowel or solid organs such as the liver, spleen, or intestines. The incidence of mechanical small-bowel obstruction caused by intraperitoneal adhesions varies from 67% to 93% [2, 1]. The presence of dense adhesion makes re-operation technically challenging because of the loss of intra-abdominal landmarks. Operations in this situation are often associated with increased bleeding, inadvertent enterotomy, and subsequent soilage of the operative field. Adhesions are a major cause of morbidity, including short-gut syndrome, chronic pain and female infertility [3, 4]. Many substances have been employed, including heparin [5], surgical barriers [6], corticosteroids [7], antihistamines [8], nonsteroidal anti-inflammatory drugs [9], fibrinolytics [10] sodium carboxymethylcellulose [11], chondroitin sulfate [12], procoagulants [9], proteolytics [13], and dextran [14]. To date, these agents have not been proven to be consistently effective, especially in the presence of inadequate hemostasis and bowel soilage.

Physical barrier and coating products have been investigated more intensely in the past several years [15, 11]. The newest physical barrier product is Interceed® (TC7), an oxidized

regenerated cellulose, which is self-adhering and absorbable [16]. Studies with Interceed® (TC7) have demonstrated that this product produced negligible tissue response and is completely absorbed from the peritoneal cavity in less than 28 days. When properly utilized, Interceed® (TC7) reduced the extent and severity of post-surgical adhesions in a clinical trial [16]. However, the use of Interceed® (TC7) in the presence of frank infection is contraindicated. For best results, the manufacturer recommends that complete hemostasis be established prior to the instillation of Interceed® (TC7) since the effectiveness of this product is reduced when it is saturated with blood.

This study was undertaken to evaluate the effectiveness of a polypentapeptide of elastin (polymer) as a physical barrier to adhesion in a contaminated abdominal wound in rats. The polymer was developed as a bioabsorbable material composed of a series of peptides which have been cross-linked by g-irradiation to form elastomeric sheets measuring approximately 2" x 2".

Several differing amino acid sequences were evaluated (unpublished data). Their success was limited by factors such as fragility and effectiveness in preventing adhesions. The polymer val-pro-gly-val-gly (poly(VPGVG)) formulation was the most promising, as it was easy to manipulate, durable and effective.

Preliminary studies indicated that this polymer, which exhibits an extraordinary biocompatibility [17], would serve as a physical barrier to adhesions in the presence of bowel soilage and hemorrhage. The emphasis of this study was to utilize the polymer in a simulated abdominal trauma model with resulting hemorrhage and contamination.

MATERIALS AND METHODS

Peptide Synthesis

The polypentapeptide is poly(Val^1-Pro^2-Gly^3-Val^4-Gly^5) which may be abbreviated as poly(VPGVG) or as any of the permutations, e.g., poly(GVGVP) wherein it is the pentamer GVGVP that is activated and polymerized. With degrees of polymerization of between 100 and 200, the end two or three residues can generally be considered as inconsequential so that any of the convenient permutations may be used in the synthesis. For secondary structural reasons of a hydrogen bond between Val^1CO and the Val^4NH, the polypentapeptide is most commonly referred to as poly(VPGVG). With either Pro or Gly at the carobxyl terminus racemization on forming the pentamer and on the polymerization of the pentamer is avoided. When the Pro residue is placed at the carboxyl terminus and nitrophenol is used in the polymerization, it has been shown to result in increased molecular weights [Urry, et al, 1985]. For this reason, the polypentapeptide was synthesized using the GVGVP permutation.

The chemical synthesis and characterization of poly(GVGVP) has been extensively reported elsewhere [18, 19]. It is to be emphasized that considerable care is required to purify and characterize the intermediates that are used in the preparation of the pentamer and in the pentamer itself which is then polymerized to make the high molecular weight polymers. Small

impurities, including racemization, that can occur as side reactions during the synthesis can produce significantly different properties in the final polymer. The component peptides Boc-VP-OBzl and Boc GVG-OBzl are each carefully synthesized and recrystallized from ether/petroleum ether and ethyl acetate/petroleum ether respectively, and coupled to give the pentamer, Boc-GVGVP-OBzl. This was hydrogenated and made to react with bis-PNPC (1.5 equiv.) in pyridine to obtain Boc-GVGVP-ONp.

Boc-GVGVP-ONp (40 g, 0.062 mole) was deblocked using TFA and a one molar solution of the TFA salt in DMSO was polymerized for 14 days using 1.6 equiv. of NMM as base. The polymer was dissolved in pyrogen-free water, dialyzed using 3500 mol. wt. cut-off dialysis tubing and lyophilized. The product was base treated with 1N NaOH (2 equiv. per pentamer), dialyzed using 50 kD mol. wt. cut-off tubing for one week and lyophilized to obtain 20.2 g (yield 79.57%) of poly(GVGVP).

The high polymer poly(GVGVP) is soluble in water below 25C. On raising the temperature, aggregation occurs with settling to form the coacervate phase. The aggregation is monitored by the onset of turbidity. The temperature for onset of turbidity provides a critical assay as to the quality of the synthesis. The correct temperature for the onset of turbidity for poly(GVGVP) is 25.5C ± 1C. ^{13}C NMR spectra to verify the

synthesis are also routinely obtained. The presence of all requisite peaks and the absence of extraneous peaks is required to verify the synthesis. This is done not only with the final product, but also with the pentamer building blocks.

Preparation of the Cross-Linked Matrix

Poly(GVGVP) is dissolved in pyrogen-free water in the concentration of 250 mg/ml. The solution is then placed in a mold and centrifuged with the temperature maintained at 10C below the transition. The temperature is then raised to 10C above the transition over a period of one hour and then centrifuged for four more hours. The coacervate phase is then checked for uniformity. If it does not have any irregularities such as bubbles, it is then g-irradiated with a 20 Mrad dose of cobalt-60 radiation to form the cross-links which result in an insoluble matrix. The molds are then opened in a laminar flow hood using sterile conditions and placed in a tube containing sterile water. The tube is then sealed until prepared for implantation.

All of the polypentapeptide material was sterilized by either heat or gas. Heat sterilization was accomplished by autoclaving in sterile saline for 20 minutes at 120C. Gas sterilization was achieved utilizing ethylene oxide and standard exposure times. The polymer was secured in gauze and a gas

permeable sealed bag for this procedure. Once sterilized, all polymer specimens were incubated at 37C for 20 minutes in .9% NaCl to allow for rehydration of the sheets prior to placement in the study animals.

Surgical Procedure

A total of 88 rats were studies with random assignment of animals to three study groups as follows: Sham-operated (n=29), polypentapeptide steam sterilized (n=30) and polypentapeptide gas sterilized (n=29). Animals were anesthetized with isoflurane administered via nose cone. A midline abdominal incision was made through the skin and muscle tissue. The cranial portion of the ileum was located. The abdominal wall muscle peritoneum was excoriated in a small area using a #15 scalpel blade until hemorrhage was noted. At this point, the animals were divided into their respective groups. In the control animals, there was no physical barrier placed between bowel loop and the abdominal wall. In the study groups, the polypentapeptide was placed directly over the excoriated area. The intestinal loop was then loosely secured to the excoriated area with 2-0 nylon (stay suture) which was tied subcutaneously in all groups. In the case of the polypentapeptide, the stay suture was placed directly through the polypentapeptide sheet to insure proper placement. Four puncture wounds were made with a 20-gauge hypodermic needle in the bowel that was apposed to the excoriated peritoneal

musculature. Intestinal contents were then milked through the puncture wounds in the bowel which contaminated the area. There was hemorrhage as a direct result of the puncture wounds. There were no attempts to acheive hemostasis. The abdominal incision was closed in a continuous pattern with 2-0 nylon and the skin with wound clips (Figure 1). Each animal was given 3cc of lactated Ringers solution subcutaneously prior to recovery from anesthesia. Animals had free access to food and water post recovery.

On day 7 post surgery, the animals were anesthetized briefly with isoflurane as before. The stay suture was surgically removed through a skin incision exposing the subcutaneously tied stay suture. The nylon suture was cut and withdrawn. The skin was closed with wound clips. At day 14, animals were euthanized with carbon dioxide via inhalation and abdominal adhesions were assessed.

Adhesions were graded according to the parameters outlined in Table 1, which are a modification of the grading system presented by Nair et al.[20]. Comparisons were made by contingency tables between treatment groups $p<0.05$.

RESULTS

Table 2 summarizes the results of the study. Significant adhesions were noted in 90% of the control rats studied. Since all control animals had at least one area of adhesion formation, we were satisfied with the method utilized for the creation of adhesions in this model. The incidence of significant adhesion was 28% for the polymer group versus 90% for control animals ($p<0.05$). There was no significant difference in polypentapeptide effectiveness based on sterilization techniques. Gas sterilization resulted in insignificant adhesions for 80% of the animals ($p<0.05$). Steam sterilized polymer provided protection for 56% of the animals ($p<0.05$). Five animals had grade 2 adhesions via a defect in the polymer. These defects probably resulted from an inadvertent tear during the placement of the stay suture. In these instances, the adhesion was strictly confined to the tear in the polymer. These animals were included in the study in the grade 2 category. During the course of the experimental design, four animals died from bowel strangulation with in 24 hours as a complication of the surgical method. These animals were not included in the study.

DISCUSSION

The pathogenesis of adhesion formation has been described [9, 21, 1, 22]. Adhesions are the most common cause of intestinal obstructions in the industiralized nations. Greater than 80% of these adhesions are the result of previous abdominal surgery [21, 23]. Post-mortem examinations reveal that at least 67% of people who have had a laparotomy developed adhesions as a direct result of this procedure. This figure rose to 93% for patients who experienced two or more procedures [1]. With these facts in mind, it is clear why this topic has been the subject of numerous studies.

Much of the postoperative adhesion prevention work has been conducted utilizing models suited for the investigation of infertility. These models are designed to minimize hemorrhage and contamination. Therefore, most of the adhesion preventing substances tested to date are not effective when there is bleeding and bowel soilage. Minimal work has been done in the area of adhesions secondary to severe abdominal trauma or insult, especially when the abdominal cavity is contaminated with intestinal contents and blood. This model was designed with this principal in mind. The polypentapeptide of elastin was effective in this contaminated peritoneal wound.

Studies evaluating synthetic, absorbable barriers to adhesion formation include Poloxamer 407, Surgicel® and

Interceed® (TC7). While Surgicel provided minimal protection against adhesions, Interceed® (TC7) has been proven to reduce the incidence and severity of postoperative adhesions. However, the efficacy of Interceed® (TC7) was significantly reduced in the presence of blood and is contraindicated in the presence of frank infection [16]. While it has been demonstrated that Poloxamer 407 may have some hemostatic properties, the effectiveness in a contaminated setting has not been studied to date.

Exposure to heat results in a change in the configuration of the proline peptide in the poly-VPGVG formulation. However, this alteration of the configuration did not alter the adhesion prevention effectiveness of polymer in this model.

The sheet form tested by this investigator was not self adherent and not absorbed within six months (unpublished observations). At the end of the two week period it was found floating freely within the peritoneal cavity. There was no gross or histological evidence of inflammation associated with the presence of the polymer. It would appear that the polymer is inert with respect to the abdominal cavity.

The polypentatpeptide in sheet form has also been examined in a rabbit strabismus surgery model in which two compositions were compared: poly(VPGVG) and poly(3{VPGVG},{VPGFG}) [24].

Neither significant inflammation or significant scarring occurred with either of the compositions, one placed surrounding the superior rectus muscle beneath the conjunctiva, where scarring always occurred in the controls. No fibrous membrane formed around poly(VPGVG) in a two-month period whereas a fibrous membrane did form around poly(3(VPGVG),(VPGFG)) within two weeks. These initial studies showed poly(VPGVG) to be promising for the prevention of adhesions in the rabbit strabismus model with the current objective being to enhance the rate of degradation. [25]

The polypentapeptide of elastin can also be formulated in liquid and foam forms. These forms were completely absorbed after 14 days in a pilot study using 10 animals (data not shown). Currently studies are being conducted using many more animals and larger quantities of these forms. This material also has the potential to serve as a method of drug-delivery to the surgical site [26, 27, 25].

In conclusion, it appears that the polypentapeptide of elastin is an effective physical barrier to surgically induced adhesions in this animal model. This model also provided a source of bleeding and frank contamination to further asses the capabilities of the polymer in a trauma setting. The polymer may potentially be very useful in the prevention of postoperative intra-abdominal adhesions in contaminated wounds.

Further studies are needed to completely evaluate the possible applications of this polypentapeptide of elastin in the area of

wound healing and adhesion prevention.

Abbreviations

Boc, tert-butyloxycarbonyl; OBzl, benzyl ester; bis-PNPC, bis[4-nitrophenyl]carbonate; ONp, p-nitrophenyl ester; TFA, trifluoroacetic acid; DMSO, dimethylsulfoxide; NMM, N-methyl morpholine; ^{13}C NMR, ^{13}C nuclear magnetic resonance; G[Gly], glycine; P[Pro], proline; V[Val], valine.

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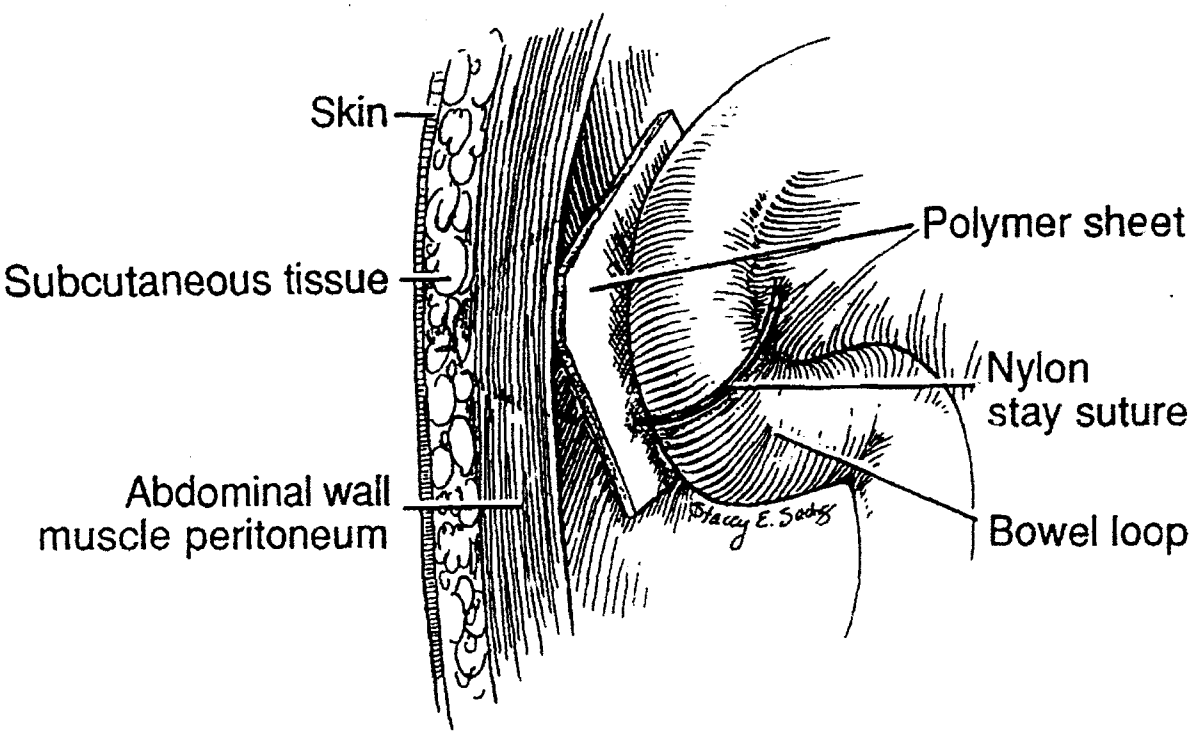


FIGURE 1 Schematic diagram of the polypentapeptide of elastin after surgical placement.

TABLE I

Scoring Criteria for Adhesions

Grade	Description	Classification
0	No Adhesions	Insignificant Adhesions
1	Single band of adhesion composed of omental fat between omentum and abdominal wall offering no resistance to separation.	
2	Involving omental fat and intestines, with a fibrous band of adhesion tissue between viscera and abdominal wall. Moderate force required for separation.	Significant Adhesions
3	Abscessed adhesion involving omental fat, abdominal wall, ± intestines with fibrous connective tissue proliferation. Sharp dissection needed for separation.	

TABLE II

Grading of Adhesions on Postmortem Examination

Grade	Control (N)	Gas (N)	Steam (N)
0	0	59(17)*	40(12)*
1	10(3)	21(6)*	16(5)*
2	62(18)	10(3)*	13(4)*
3	28(8)	10(3)*	30(9)*

*P<.05, as compared to control. (N) refers to number of rats studied

LEGEND

Figure I Schematic diagram of surgically implanted polypentapeptide of elastin.

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Elastomeric polytetrapeptide matrices: Hydrophobicity dependence of cell attachment from adhesive $(GGIP)_n$ to nonadhesive $(GGAP)_n$ even in serum

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The cross-linked polytetrapeptide matrices based on the repeating amino acid sequences, GGAP, GGVP and GGIP, were prepared and tested for cell adhesion promoting activity in both the absence and presence of fetal bovine serum. For comparison, X^{20}-poly(GVGVP), a matrix previously shown to be a poor support for cell attachment and spreading, was included. In the absence of serum, all three polytetrapeptide-based matrices and the polypentapeptide-based matrix were negative for the adhesion of fibroblasts and endothelial cells. In the presence of serum, various sub-maximal levels of cell adhesion were found for all matrices except for the matrix based on GGAP. An apparent correlation was noted between the degree of cell attachment to the different polytetrapeptide-based matrices and the hydrophobicity of those matrices where increased hydrophobicity results in increased cell attachment. The property of being refractory to ligamentum nuchae fibroblast and human umbilical vein endothelial cell adhesion in the presence of serum indicates a potential use for X^{20}-poly(GGAP) in the development of, for example, additional physical barriers for the prevention of post-surgical and post-trauma adhesions.© 1993 John Wiley & Sons, Inc.

INTRODUCTION

Elastomeric polypeptide matrices are a new group of synthetic biomaterials which have considerable potential for use in medical and other applications.[1] These materials originally based on the repeating sequences of elastin are now being constructed from modified sequences giving consequently modified matrix properties. They consist of polypeptides, comprising repeated oligopeptides of given sequence, which are cross-linked by gamma irradiation.[2] Depending largely on the repeating sequence, such matrices can be prepared having a wide range of elastic moduli.[3] The matrices can be cast into various shapes including tubes and sheets,[1] and some have now been determined to be biocompatible.[4] In particular, poly(GVGVP) and its 20 Mrad γ-irradiation cross-linked matrix, X^{20}-poly(GVGVP), have been subjected to 11 biological tests: 1) the Ames mutagenicity test, 2) cytotoxicity-agarose overlay, 3) acute systemic toxicity, 4) intracutaneous toxicity, 5) muscle implantation, 6) acute intraperitoneal toxicity, 7) systemic antigenicity, 8) dermal sensitization—the Magnusson and Kligman maximization method, 9) pyrogenicity, 10) Lee White clotting study, and 11) *in vitro* hemolysis test—all of which showed remarkable biocompatibility.[4] Also, the series of tests are under way for poly(GGAP) and X^{20}-poly(GGAP). About two-thirds of the tests are completed with similar results (unpublished).

Investigation of the cellular interactions of these materials is central to the development of biomedical applications. The X^{20}-poly(GVGVP), the 20 Mrad γ-irradiation cross-linked matrix based on the repeating pentamer VPGVG of elastin, has been shown to be a poor support for cell adhesion in the absence of serum.[5] In the presence of serum submaximal levels of cell adhesion are found although the matrix is poorly supportive of cell growth. The X^{20}-poly(GVGVP) modified by the covalent incorporation of RGDS, the major cell attachment site of fibronectin, has been shown to support maximal cell adhesion in the absence of serum and to be an excellent support for cell growth in the presence of serum.[5] Thus, modulation of the cell adhesive interaction with the

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elastomeric matrix by the incorporation of adhesion sequences is a viable option. However, owing to the limited cell adhesion which does occur in the presence of serum to X^{30}-poly(GVGVP), a more ideal matrix could yet be sought in which to insert active sequences, and thereby to direct specific matrix utilization.

The work reported herein describes the synthesis and study of the cell adhesion supporting properties of three new matrix materials based on repeating tetrapeptides. One of these, X^{20}-poly(GGAP), was found to be completely nonsupportive of ligamentum nuchae fibroblast and human umbilical vein endothelial cell adhesion, both in the absence and presence of serum. Should this non-cell adhesive property be general for all cell types, then many potential applications may be envisaged.

MATERIALS AND METHODS

Synthesis of Polytetrapeptides

The synthesis and cross-linking of poly(GVGVP) have been previously described.[2,4,6,7] The syntheses of poly(GGVP) and poly(GGIP) have also been previously described.[8,9] The tetrapeptide, Boc-GGAP-OH was synthesized by the stepwise solution phase method. The protected peptides were characterized by carbon-13 nuclear magnetic resonance before polymerization to verify structure and purity. Thin layer chromatography (TLC) was performed on silica gel plates obtained from Whatman, Inc., with the following solvent systems: R_f^1, $CHCl_3$:CH_3OH:CH_3COOH (85:15:3); R_f^2, $CHCl_3$:CH_3COOH (90:10:3); R_f^3, $CHCl_3$:CH_3OH:CH_3COOH (85:15:3). The compounds on TLC plates were detected by UV light by spraying with ninhydrin or chlorine/tolidine. All Boc amino acids and HOBt were purchased from Advanced ChemTech, (Louisville, KY). EDCI was obtained from Bachem, Inc. (Torrance, CA).

Boc-Ala-Pro-OBzl (I)

To Boc-Ala-OH (56.76 g, 0.3 mole) dissolved in acetonitrile (500 mL) and cooled to 0°C was added NMM (32.97 mL). The solution was cooled −15° ± 1°C and isobutyl chloroformate (41.01 mL) was added slowly under stirring while maintaining the temperature at −15°C. After stirring the reaction mixture for 10 min at this temperature, HOBt (40.56 g, 0.3 mole) was added. The reaction mixture was stirred for an additional 10 min and a precooled solution of HCL · H-Pro-OBzL (72.5 g, 0.3 mole) and NMM (32.97 mL) in DMF (600 mL) was added slowly. After 20 min, the pH of the solution was adjusted to 8 by the addition of NMM and the reaction was continued overnight. The solvent was removed under reduced pressure and the residual DMF solution was poured into about 2000 mL of ice-cold 90% saturated $KHCO_3$ solution and stirred for 30 min. Since the peptide did not precipitate out, it was extracted into $CHCL_3$, which was washed with water, 20% citric acid, and water, and dried over Na_2SO_4, and the solvent was removed under reduced pressure. The resulting oil was recrystallized from ether/petroleum ether. The crystals were filtered, washed with petroleum ether, and dried to obtain 79.9 g (yield, 70.75%) of I. R_f^1, 0.5; R_f^2, 0.61.

Boc-Gly-Ala-Pro-OBzl (II)

Boc-Ala-Pro-Bzl (75.3 g, 0.2 mole) was deprotected by stirring for 1.5 h. in 4.2 N HCl in dioxane. Excess HCl and dioxane were removed under reduced pressure, triturated with ether, filtered, washed with ether, and dried (yield 100%).

A solution of Boc-Gly-OH (35.04 g, 0.2 mole) and HOBt (27.02 g, 0.2 mole) in DMF was cooled to −15°C with stirring and EDCI (38.34 g, 0.2 mole) was added. After 20 min, a precooled solution containing the above hydrochloride salt and NMM (21.98 mL, 0.2 mole) was added and the reaction mixture was stirred overnight at room temperature. The mixture was evaporated to a thick oil which was dissolved in $CHCl_3$. This solution was extracted with water, 10% citric acid, water, 5% $NaHCO_3$, water, and dried over Na_2SO_4. The solvent was removed under reduced pressure and the resulting oil was crystallized from ether/petroleum ether. The solid was filtered, washed with petroleum ether, and dried to obtain 76.2 g (yield 87.88%) of II. R_f^1, 0.39; R_f^2, 0.44.

Boc-Gly-Gly-Ala-Pro-OBzL (III)

Compound II (65.03 g, 0.15 mole) was deblocked with HCl/dioxane and coupled to Boc-Gly-OH (26.3 g, 0.15 mole) using EDCI with HOBt in the same manner as that described for II to give 63.2 g (yield 85.9%) of III R_f^1, 0.3; R_f^2, 0.46.

Boc-Gly-Gly-Ala-Pro-ONp (IV)

Boc-Gly-Gly-Ala-Pro-OBzl (30.0 g, 0.061 mole) was dissolved in glacial acetic acid (300 mL) and 3.0 g of 10% Palladium on activated charcoal was added. This mixture was hydrogenated at 40 psi for 6 h. The reaction mixture was filtered through celite and the solvent was removed in vacuum. The resulting residue was triturated with ether, filtered, washed with ether, and dried to obtain Boc-Gly-Gly-Ala-Pro-OH.

This acid was dissolved in pyridine (200 mL) and reacted with Bis-PNPC (1.5 equiv.). When the reaction was completed, as monitored by TLC, the solvent

was removed under reduced pressure. The residue was taken into $CHCl_3$ and washed with water, 10% citric acid, water, 5% $NaHCO_3$, water, and dried over Na_2SO_4. The solvent was removed under reduced pressure, triturated with ether, filtered, washed with ether, and dried to obtain 18.3 g (yield 57.52%) of IV. R_f^1, 0.22; R_f^2, 0.38; R_f^3, 0.48.

Poly(Gly-Gly-Ala-Pro) (V)

Boc-Gly-Gly-Ala-Pro-ONp (18.0 g, 0.035 mole) was deblocked using TFA and a one-molar solution of the TFA salt in DMSO was polymerized for 14 days using 1.6 equiv. of NMM as base. The polymer was dissolved in pyrogen-free water, dialyzed using 3500 mol. wt. cut-off dialysis tubing and lyophilized. The product was base-treated with 1 N NaOH (2 equiv. per tetramer), dialyzed using 50 kD mol. wt. cut-off tubing for 8 days and lyophilized to obtain 5.02 g (yield 51.5%) of V.

The purity of the intermediate and of the final product was checked by TLC, carbon-13 NMR spectroscopy, and amino acid analysis. The presence of all requisite peaks and the absence of extraneous peaks are required to verify the synthesis.

Preparation of the Cross-linked Matrices

Poly(GGVP) and poly(GGAP) were dissolved separately in pyrogen-free water in the concentration of 600 mg/mL. The solutions were then placed in a mold and centrifuged with the temperature maintained at 25°C for 1 h. The temperature was then raised to 40°C and centrifuged for 8 more h. Poly(GGIP) was also dissolved in pyrogen-free water in the concentration of 400 mg/mL. The solution was centrifuged with the temperature maintained at 10°C over a period of 5 h and 30°C over a period of 15 h. The coacervate phase was then checked for uniformity. If they did not have irregularities such as bubbles, they were then γ-irradiated with a 20 Mrad dose of cobalt-60 radiation to form the cross-links which resulted in an insoluble matrix. The molds were then opened in a laminar flow hood using sterile conditions and placed until use in PBS containing 100 μ/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin-B at 4°C.

Cells

Bovine ligamentum nuchae fibroblasts (LNFs) were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 μ/mL penicillin, and 100 μg/mL streptomycin as previously described.[10]

Human umbilical vein endothelial cells (HUVECs) at passage 1 were bought from Endotech Corporation, (Indianapolis, IN). Cells were cultured in Medium 199 containing 25 mM HEPES, 20% fetal bovine serum 2 mM L-glutamine, 13.2 μL/mL Endo-Ret HI-GF (Endotech) endothelial cell growth supplement, 100 μ/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin-B. Tissue culture flasks for HUVECs were precoated with 2% bovine gelatin (Sigma) at 7.5 μL/cm².

Both LNFs and HUVECs were passaged by trypsinization and were used for experiment at passage 8 or earlier.

Adhesion Assay

A detailed description of the assay and apparatus has been given previously.[5] Cells were harvested using 0.05% trypsin/0.53 mM EDTA (Gibco) and then treated with 0.2 mg/mL soybean trypsin inhibitor. The cell density plated on the test matrices was 10 cells/mm². Cell concentration was adjusted to 0.32×10^5/mL and 50 μL aliquots were plated into glass cylinders containing 50 μL of appropriate medium over discs of the test matrices. Three replicates were used for each experimental condition. The apparatus was incubated for 3 or 20 h to allow cell adhesion, after which the matrices were gently rinsed in PBS, pH 7.2, and then fixed in 3.5% paraformaldehyde in PBS, pH 7.2. Phase contrast microscopy was then used to count and classify the adherent cells. The area of one field of view counted was 1.2 sq. mm. and five such fields were counted for each replicate. The total area plated over was 15.9 sq. mm. for each replicate. Thus, 37% of the total area plated was counted. The target plated cell density of 100 cells/sq. mm. was chosen as suitable for individual cell recognition, quantitation, and classification. To normalize variations in the actual density of cells plated between experiments, the results are given as the percent of the appropriate positive control.

RESULTS

To examine the adhesion of LNFs and HUVECs to the cross-linked elastomeric matrices alone, the cells were plated onto the test matrices in the absence of serum and in the presence of 0.1% BSA. After incubation for 3 h the matrices and control substrata were rinsed and then fixed, as described in Materials and Methods. It was found (Fig. 1A) that neither cell type was able to adhere to any of the three polytetramer-based matrices and that cell adhesion to X^{20}-poly(GVGVP) was extremely poor, as had been previously described.[5] The few fibroblasts or endothe-

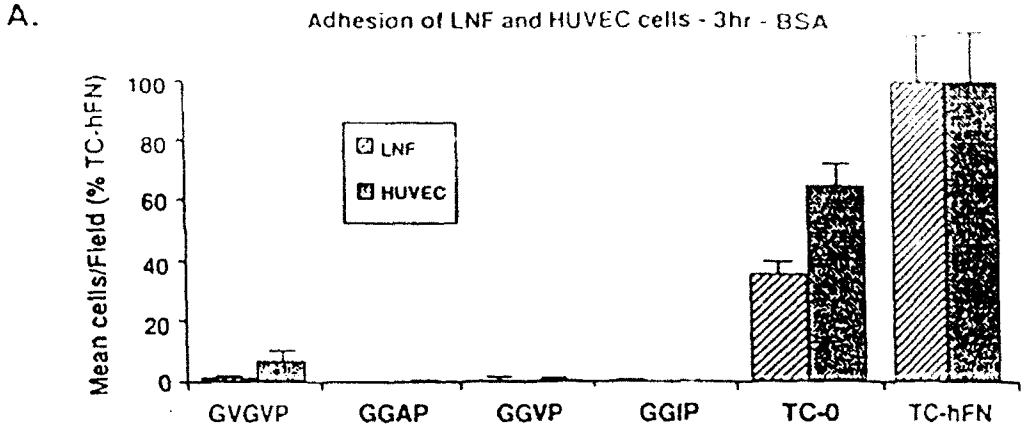


Figure 1A. The mean number of LNFs and HUVECs adhering to X^{20}-poly-(GGAP), X^{20}-poly(GGVP), X^{20}-poly(GGIP), and X^{20}-poly(GVGVP) matrices after 3 h in the presence of 0.1% BSA. TC-O and TC-hFN represent uncoated and human fibronectin coated (10 μg/mL) tissue culture plastic substrata. The vertical bars represent one standard error.

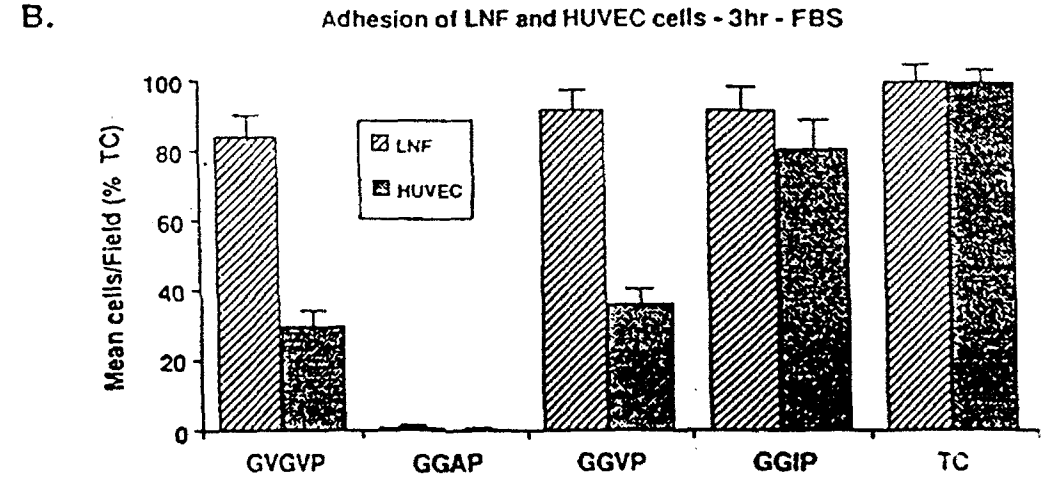


Figure 1B. The mean number of LNFs and HUVECs adhering to X^{20}-poly-(GGAP), X^{20}-poly(GGVP), X^{20}-poly(GGIP), and X^{20}-poly(GVGVP) matrices after 3 h in the presence of 10% fetal bovine serum (LNFs) and 20% fetal bovine serum (HUVECs). TC represents normal tissue culture plastic substratum. The vertical bars represent one standard error.

lial cells which were found adherent to the matrices were rounded and had not spread (Figs. 2 and 3).

In the presence of 10% fetal bovine serum, LNFs were found to adhere to X^{20}-poly(GGIP), X^{20}-poly(GGVP), and X^{20}-poly(GVGVP) at near control tissue culture substratum levels after 3 h incubation (Fig. 1B). Also, on these matrices the fibroblasts were largely well spread (Fig. 2). The fibroblasts failed to attach or spread on the X^{20}-poly(GGAP) after 3 h in the presence of serum (Figs. 1B and 2). The results for HUVECs plated in the presence of 20% fetal bovine serum (Figs. 1B and 3) were similar to those for LNFs except that the relative levels of cell attachment to X^{20}-poly(GGVP) and X^{20}-poly(GVGVP) were much less. The level of attachment of HUVECs to X^{20}-poly(GGIP) was only slightly less than that found for LNFs. No endothelial cell adhesion to X^{20}-poly(GGAP) was found (Figs. 1B and 3).

After 20 h of incubation in the presence of serum (Figs. 1C, 2 and 3), the results found for both LNFs and HUVECs were essentially the same as for 3 h incubation except that the relative levels of cell attachment to the matrices were generally reduced. Again, for both LNFs and HUVECs, the X^{20}-poly(GGAP) gave no support for cell adhesion.

The mean residue hydrophobicity of the polytetrapeptide matrices based on the temperature of inverse temperature transition hydrophobicity scale[11] are 37°C for GGAP, 31.5°C for GGVP, and 28°C for GGIP. The lower the temperature the greater the

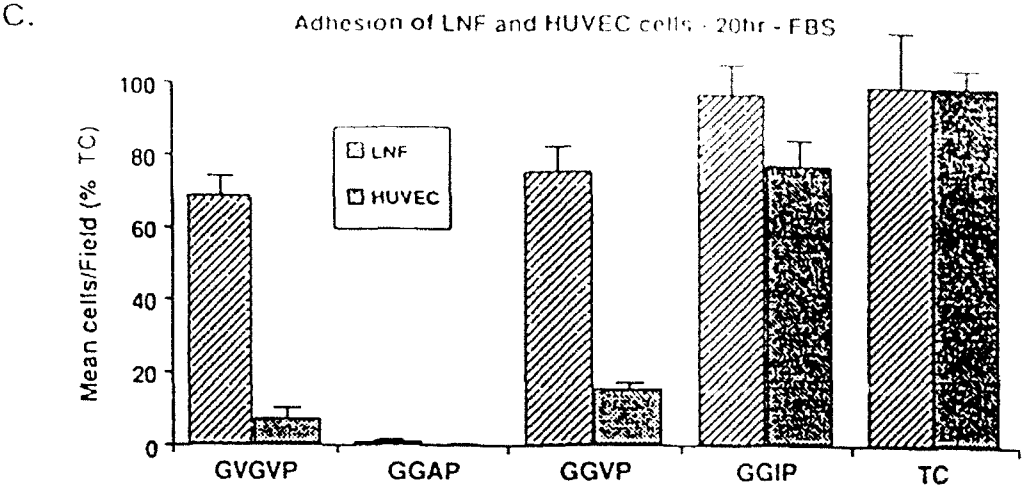


Figure 1C. The mean number of LNFs and HUVECs adhering to X^{20}-poly-(GGAP), X^{20}-poly(GGVP), X^{20}-poly(GGIP) and X^{20}-poly(GVGVP) matrices after 20 h in the presence of 10% fetal bovine serum (LNFs) and 20% fetal bovine serum (HUVECs). TC represents normal tissue culture plastic substratum. The vertical bars represent one standard error.

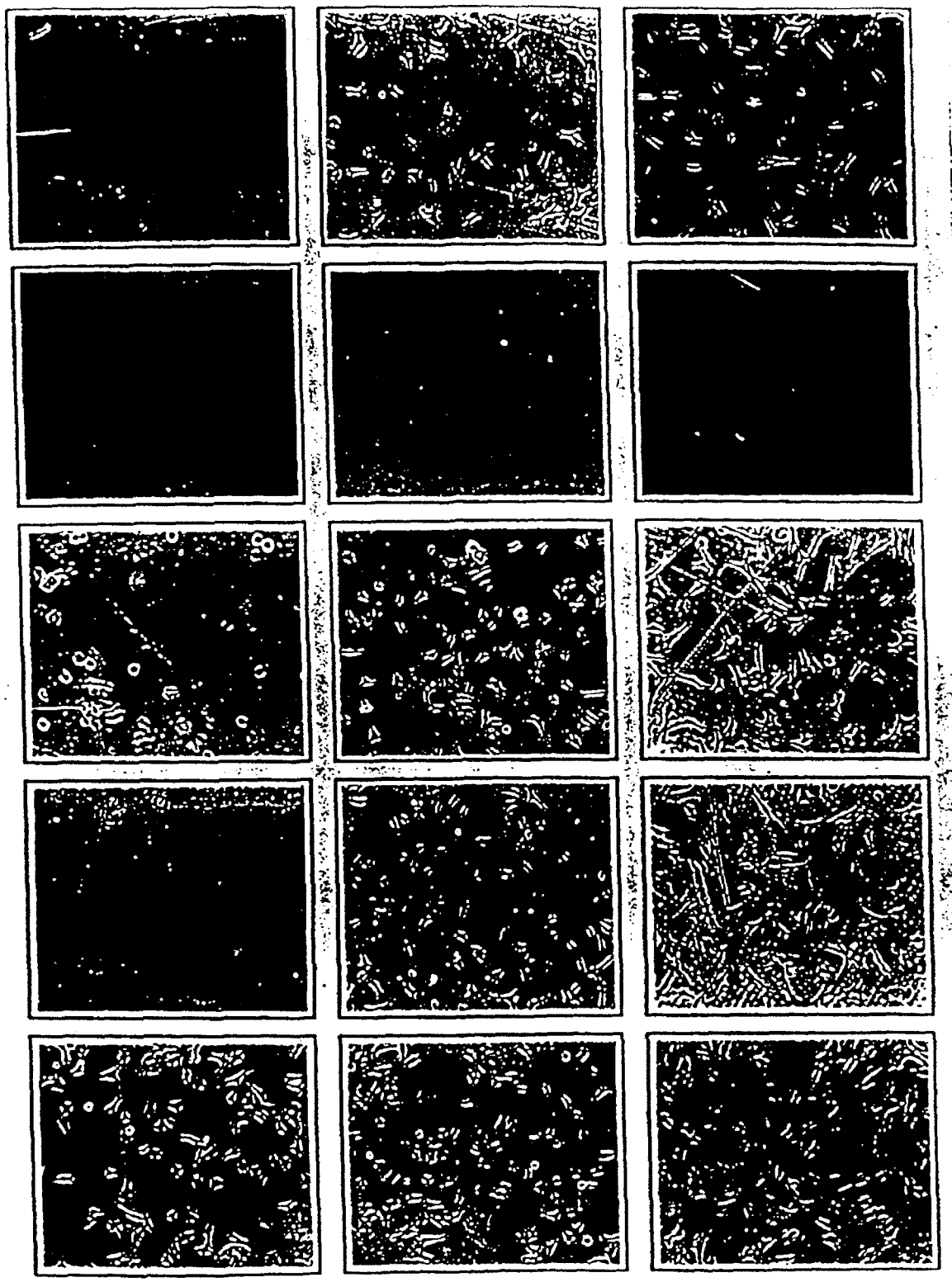
hydrophobicity. It can be seen from Figures 1B and 1C that, of the three polytetrapeptide-based matrices in the presence of serum, the least hydrophobic (GGAP) showed the lowest levels of cell adhesion and the most hydrophobic (GGIP) showed the highest levels of cell adhesion. The correlation coefficients for these relationships were −0.92, −0.98, −0.98, and −0.90 for 3 h and 20 h of LNF and 3 h and 20 h of HUVEC attachment, respectively.

DISCUSSION

The X^{20}-poly(GVGVP) matrices have been found to be effective in the prevention of surgically induced adhesions in the rabbit eye[12] and also in a contaminated, bloody, peritoneal cavity model.[13,14] In the latter study with 29 control rats and 29 test rats using ethylene oxide sterilization of X^{20}-poly(GVGVP), there were significant adhesions 90% of the time for the control animals, and there were no (59%) or insignificant (21%) adhesions for 80% of the test animals. Nevertheless, the finding that, in the presence of serum *in vitro* considerable but submaximal cell attachment to X^{20}-poly(GVGVP) occurs,[5] whereas no cell attachment occurs to X^{20}-poly(GGAP) even in 20% serum, suggests that X^{20}-poly(GGAP) would also be an effective physical barrier for the prevention of adhesions and perhaps even more effective when there is much serum present, and that in the presence of much serum a greater functional specificity could be achieved when adding specific functional sequences to poly(GGAP).

The previous finding that X^{20}-poly(GVGVP) provides a relatively poor support for cell growth in serum should be viewed in relation to the *in vivo* implant studies. It is significant, even after several months in the rat peritoneal cavity[13,14] and after two months beneath the conjunctiva of the rabbit eye, that there is no fibrous capsule formation around X^{20}-poly(GVGVP). The absence of a fibrous capsule formation taken together with the biocompatibility results suggest that X^{20}-poly(GVGVP) is ignored by the body. This is also consistent with the results of Picciolo et al.,[15] which, when studying the inflammatory reaction of elutriated human monocytes as followed by the stimulation of reactive oxygen production, showed X^{20}-poly(GVGVP) either to elicit no effect or to lower slightly the background oxidative bursts.

The finding reported herein that X^{20}-poly(GGAP) does not support cell adhesion even in the presence of 20% serum is thus relevant to the development of a surgical adhesion preventative biomaterial, for example, for cardiopulmonary bypass procedures where there may be substantially more bleeding than in the contaminated, bloodied peritoneal cavity model. Relevant to other applications, the X^{20}-poly(GVGVP) containing the covalently incorporated RGDS cell adhesion sequence of fibronectin has been previously shown to support the adhesion of cells in the presence of BSA alone, whereas X^{20}-poly(GVGVP) itself does not.[5] Under these conditions the RGDS sequence behaves as a ligand for the vitronectin receptor.[16] Because of the cell adhesion to X^{20}-poly(GVGVP) found in the presence of serum, testing of cell interactions with incorporated adhesion sequences under conditions relevant to high serum was not possible. Now X^{20}-poly(GGAP) may provide a suitable nonadhesive matrix for the testing of incorporated adhesion promoting sequences in the presence of serum with-



igure 2. The adhesion of LNFs to X^{30}-poly(GVGVP), row 1; X^{30}-poly-(GGAP), row 2; X^{30}-poly(GGVP), row 3; X^{30}-poly(GGIP), row 4; and to tissue culture plastic, row 5; (fibronectin precoated only in the 0.1% BSA condition) in the presence of 0.1% BSA for 3 h, column 1; 10% fetal bovine serum for 3 h, column 2; and in the presence of 10% fetal bovine serum for 20 h, column 3.

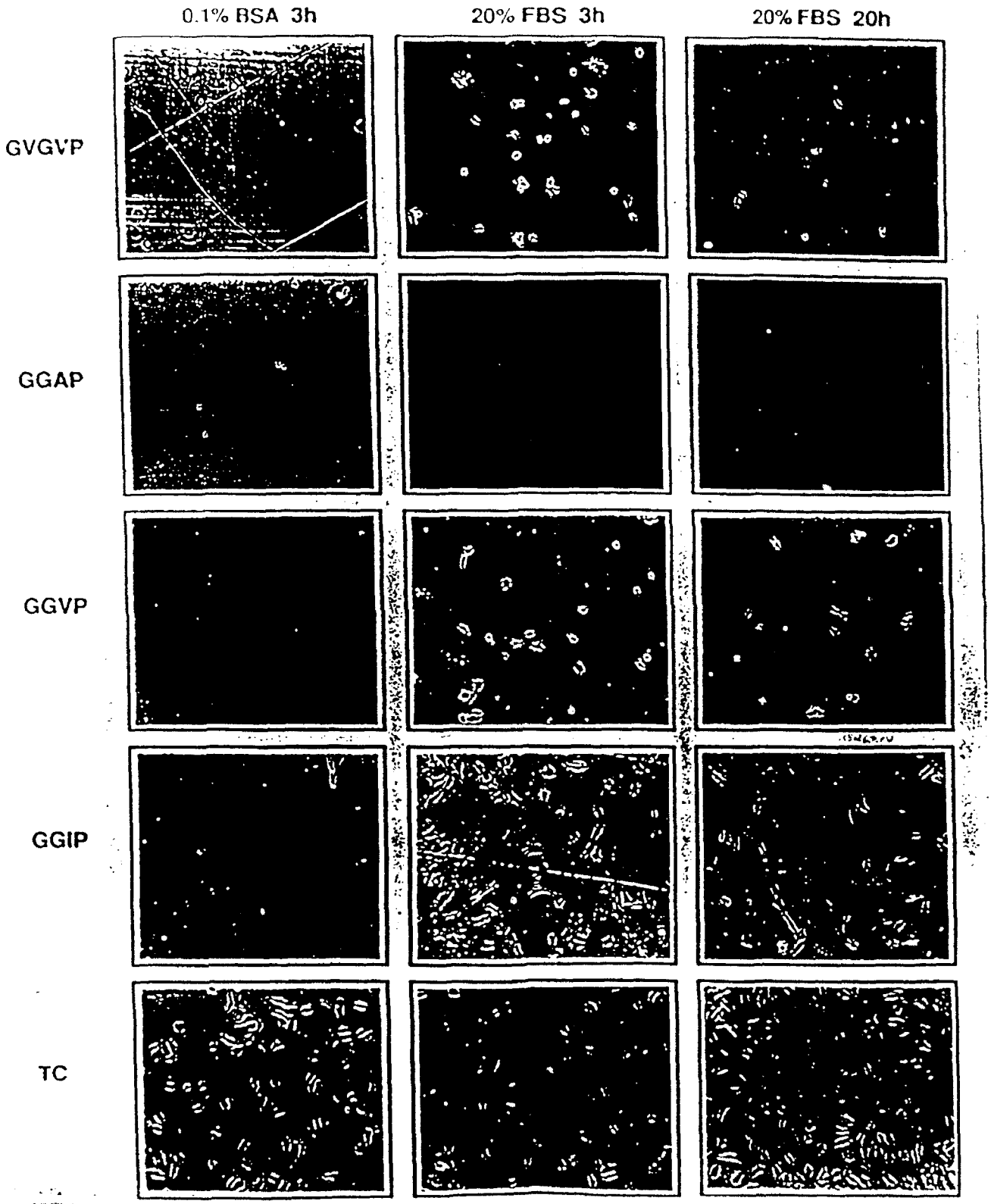


Figure 3. The adhesion of HUVECs to X^{20}-poly(GVGVP), row 1; X^{20}-poly-(GGAP), row 2; X^{20}-poly(GGVP), row 3; X^{20}-poly(GGIP), row 4; and to tissue culture plastic, row 5; (fibronectin precoated only in the 0.1% BSA condition) in the presence of 0.1% BSA for 3 h, column 1; 20% fetal bovine serum for 3 h, column 2; and in the presence of 20% fetal bovine serum for 20 h, column 3.

out the background interference of serum protein binding resulting in cell adhesion. Another relevant application could be the intimal lining of a vascular prosthesis.

The apparent decrease of both HUVEC and LNF adherent cell numbers on some matrices between 3 and 20 h in the presence of serum appears to be largely a result of the differential cell proliferation which would be expected between adhesive and poorly adhesive substrata.[17] Whereas the cells on the positive control substrata or on matrices containing RGDS adhesion sequences[5] proliferate normally, cells on more poorly adhesive substrata as indicated by the levels of adhesion at 3 h do not. The poorer the adhesion at 3 h, the lower the levels of proliferation, as indicated by the lower numbers of attached cells relative to the positive control at 20 h.

The cell adhesion to X^{20}-poly(GVGVP) in the presence of serum is almost certainly due to the association or adsorption of adhesion proteins such as fibronectin and vitronectin from the serum, as occurs with other biomaterials.[18–21] Equally, the lack of cell adhesion to X^{20}-poly(GGAP) in the presence of serum would seem to be due to the lack of association or adsorption of adhesion promoting serum components. The observed correlation of increased cell adhesion with increased hydrophobicity of the polytetrapeptide-based matrices in the presence of serum suggests that the serum components responsible may associate by hydrophobic interactions, and that at a certain reduced level of matrix hydrophobicity [ie, that of X^{20}-poly(GGAP)] their association is insufficient to support cell attachment. This finding thus suggests a hydrophobicity modulation strategy for the development of further adhesion nonsupportive surfaces.

Any strategy for controlling cell adhesion involving hydrophobicity, however, requires an appropriate measure of hydrophobicity. Of course, there have been many studies of cell attachment to biomaterials; these have used indices relevant to hydrophobicity such as contact angle and wettability,[22] equilibrium water content,[23] and further considerations of surface tension and surface free energy,[24–26] and there is a general perspective that increased hydrophobicity enhances cell adhesion.[27–31]

As early appreciated by Vroman and Adams,[18] "... the concepts of wettability cannot even be applied to water permeable membranes." This includes X^{20}-poly(GVGVP), which is about 60% water by weight at physiological temperatures[32] and X^{20}-poly(GGAP) which, as the poly(GGVP) coacervate, contains even more water.[33] It is possible to invoke a new measure of hydrophobicity, however, which is relevant to both of these membranes and to the potential membrane binding serum proteins as well. It is a hydrophobicity scale for polypeptides and proteins based on the temperature, T_t, of the inverse temperature transition for hydrophobic folding.[34] This T_t-based hydrophobicity scale is particularly relevant to these membranes as it was developed using polypeptides of the general formula, poly[f_v(GVGVP, f_x(GXGVP)] where f_v and f_x are mole fractions with $f_v + f_x = 1$, and where X can be any amino acid residue or a chemical modification thereof. Plots of f_x vs. T_t are essentially linear to $f_x = 0.5$ and the values are extrapolated linearly to $f_x = 1$ for relative values of T_t. This is the scale that was used in the hydrophobicity analysis of the Results section, which showed the correlation between hydrophobicity and cell adhesion in serum, and which showed X^{20}-poly(GGAP) to be the least hydrophobic. The perspective with the more hydrophobic membranes is that serum proteins such as vitronectin and fibronectin partially unfold to present nonpolar surfaces for hydrophobic association with the membranes.

Accordingly, two strategies for optimizing cell attachment could be considered: one in which a membrane hydrophobicity were sought which optimized, for example, vitronectin and/or fibronectin association, and one which utilized a membrane incapable of hydrophobic association with serum proteins and then introduced the appropriate cell attachment sequences into that membrane. The latter strategy has been employed by Massia and Hubbell[35] using glycophase glass, polyethylenetetraphthalate (PET), and polytetrafluoroethylene (PTFE) with attachment of the RGD-containing cell attachment sequence of fibronectin and the putative YIGSR-containing cell attachment sequence from laminin. The latter strategy is also available to X^{20}-poly(GGAP) with the incorporation of appropriate cell attachment sequences: for example, X^{20}-poly[50(GGAP), (GRGDSP)]. In particular if it also functioned as a vitronectin-like sequence, this could provide an inner lamina for a vascular prosthesis which could promote specific cell attachment while in contact with blood; it could function elastically; and it could be remodeled.

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To our knowledge these elastic protein-based polymers discussed here are the only ones presently known to have the favorable combination of properties—of biocompatibility without fibrous encapsulation; of elasticity; of potential for slow biodegradation (remodeling); of being refractory to cell attachment; and of being modifiable to achieve cell adhesion, cell spreading, and growth to confluence.

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